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Supplementary Information for

Isolation and characterization of *Helicobacter suis* from human stomach

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Supplementary Information Text

Supplemental Materials and Methods

MIC measurements

Susceptibility testing of *H. suis* isolates was performed by a broth microdilution method using NHPH medium containing triphenyl tetrazolium chloride (50 mg/L for NHP19-4003, NHP19-4004, and SNTW101c; and 10 mg/L for NHP19-4022 and NHP19-0020). Bacterial suspensions (10^5 cells/mL) were incubated with serially diluted antimicrobial agents (amoxicillin, oxacillin, clarithromycin, minocycline, gentamicin, and levofloxacin: Eiken, Tokyo, Japan; metronidazole: Sigma), and absorbance at 560 nm was measured after incubation for 8–12 days, depending on the growth of each isolate. The MIC values were defined as the lowest concentrations that inhibited the bacterial growth.

Determination of relative *H. suis* bacterial number in mice stomach

Relative *H. suis* bacterial number in the mouse stomach was evaluated by probe-based quantitative PCR targeting the gene encoding the *H. pylori* VacA-like autotransporter protein (NHP194003_11930 gene in NHP19-4003) in *H. suis*; the comparative ΔCt method was used to calculate relative gene abundance. Because other *Helicobacter* species have similar autotransporter proteins, a set of primers and probe was designed against the region specific for *H. suis* by comparing the NHP194003_11930 gene with other genes encoding autotransporter proteins, including *omp897* of *H. felis* ATCC49179; *omp1914* of *H. bizzozeronnii* M7; and *faa* (HP0610), *imaA* (HP0289), and *vlpC* (HP0922) of *H. pylori* 26695. Finally, the following primer sets were purchased from PrimeTime qPCR probes (IDT, Integrated DNA Technologies) for amplification of the NHP194003_11930 gene: NHP194003_11930_forward (5'-CTGGTAATGCATCATTAGAAGCAAA-3'), NHP194003_11930_reverse (5'-GATGGGCGCTTCTGGTTTA-3'), and NHP194003_11930_probe (/56-FAM/TGTACACAC/ZEN/CAAACAGATGAGCCGT/3IABkFQ/). β-actin was used as an internal control using following primers: β-actin_forward (5'-GACTCATCGTACTCCTGCTTG-3'), β-actin_reverse (5'-GATTACTGCTCTGGCTCCTAG-3'), and β-actin_probe (/56-FAM/CTGGCCTCA/ZEN/CTGTCCACCTTCC/3IABkFQ/). DNA was extracted from mouse stomach using the DNeasy Blood & Tissue Kit (Qiagen), and 100 ng/μL DNA was used for each reaction. PCRs were performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Probe-PCR was performed in triplicate for each sample, and the $2^{-\Delta CT}$ $_{(NHP194003\_11930\text{-}b\text{-}actin)}$ value of each sample was used for comparison. The relative value was calculated by dividing the $2^{-\Delta Ct\ (NHP194003\_11930\text{-}b\text{-}actin)}$ of each sample by the average of the $2^{-\Delta CT}$ $_{(NHP194003\_11930\text{-}b\text{-}actin)}$ values of the SNTW101c-infected group.

Measurements of cytokines and chemokines in mice serum

IFN-γ, IL-1β, IL-2, IL-4, IL-8, IL-10, IL-12 (p70), IL-17, MCP-1, RANTES, and TNF-α in serum were measured using the Milliplex MAP Mouse Cytokine/Chemokine magnetic bead panel (Millipore) on a Luminex 200 system (Luminex, Austin, TX, USA).

ELISA for measuring *H. suis*–specific IgG in mice serum

Nunc-Immuno 96-well microtiter plates (No. 439454; Thermo Fisher Scientific) containing 100 μL (4 μg/mL in 0.1 M carbonate/bicarbonate buffer, pH 9.4) of whole-cell lysates of *H. suis* SNTW101c, or BSA, were incubated overnight at 4°C in a humid covered box. After three washes with phosphate-buffered saline containing 0.05% (v/v) Tween 20, pH 7.4 (PBS-T), the wells were saturated with 200 μL of PBS containing 1% BSA for an hour at 37°C. After three washes with PBS-T, the wells were filled with 50 μL of serum samples (dilution range of 1:500 or 1:1,000 with PBS containing 1% BSA), and the plates were incubated for an hour at 37°C. After three washes with PBS-T, the wells were filled with 50 μL of a donkey anti-mouse IgG (Jackson) solution diluted 1:100,000 with PBS containing 1% BSA, and the plates were incubated for an hour at 37°C. After three washes with PBS-T, the wells were filled with 50 μL of KPL SureBlue TMB Microwell Peroxidase Substrate (1-Component) (Sera Care Life Sciences, Milford, MA, USA). The color reaction was stopped by addition of 50 μL of 1 N hydrochloric acid into the wells. The

absorbance at wavelength 450 nm (reference wavelength, 630 nm) was measured on a microplate reader.

Determination of SNPs found in *H. suis* draft genomes recovered from experimental mice infection.

Whole-genome sequencing of *H. suis* isolates recovered from mouse stomachs 4 months after infection was performed on a MiniSeq platform (Illumina) with the High Output Reagent Kit (300 cycles). The library (paired-end, insert size of 500–900 bp) was prepared using the Nextera XT DNA Library Prep Kit (Illumina). Reads were mapped to the reference genome, and then SNV and short indels were detected using CLC Genomic Workbench v11.0.1 (Qiagen).

Statistics

Prism 8 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. Data represent means ± standard deviation (SD). $p<0.05$ was considered statistically significant.

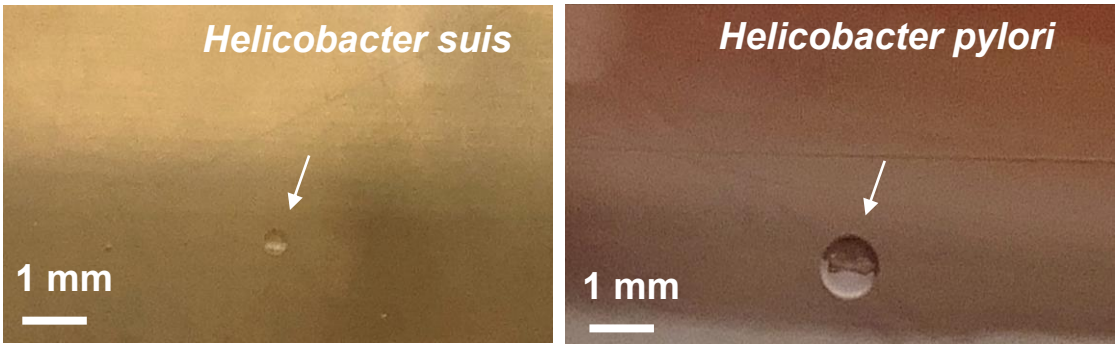

Fig. S1. Colonies of *Helicobacter suis* strain NHP19-4022 from a human patient and *Helicobacter pylori* strain TS1903 on an agar plate

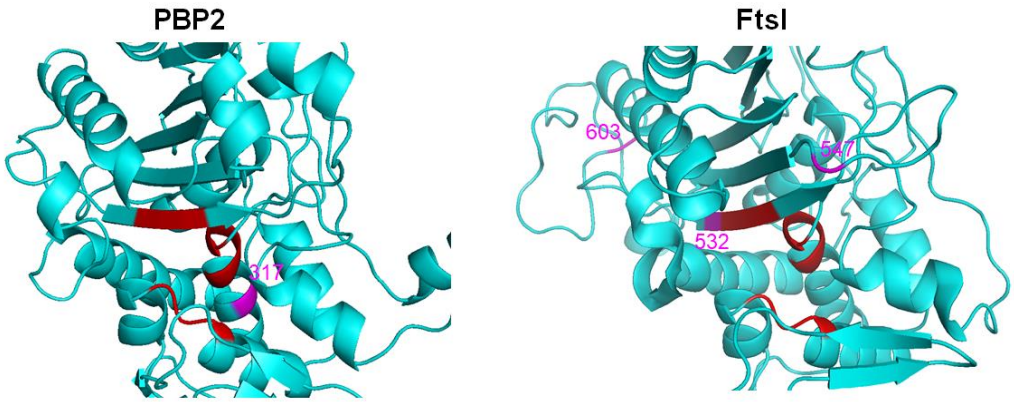

Fig. S2. Predicted structures of PBP2 and FtsI of *H. suis* SNTW101c

Structures were predicted using Robetta (http://robetta.bakerlab.org/).

Red region; penicillin-binding motif (SVVK-SVD-KTG), pink positions; specific substitutions (S317M in PBP2 and G532A, E547K, and T603I in FtsI) found in *H. suis* NHP19-4004 strain.

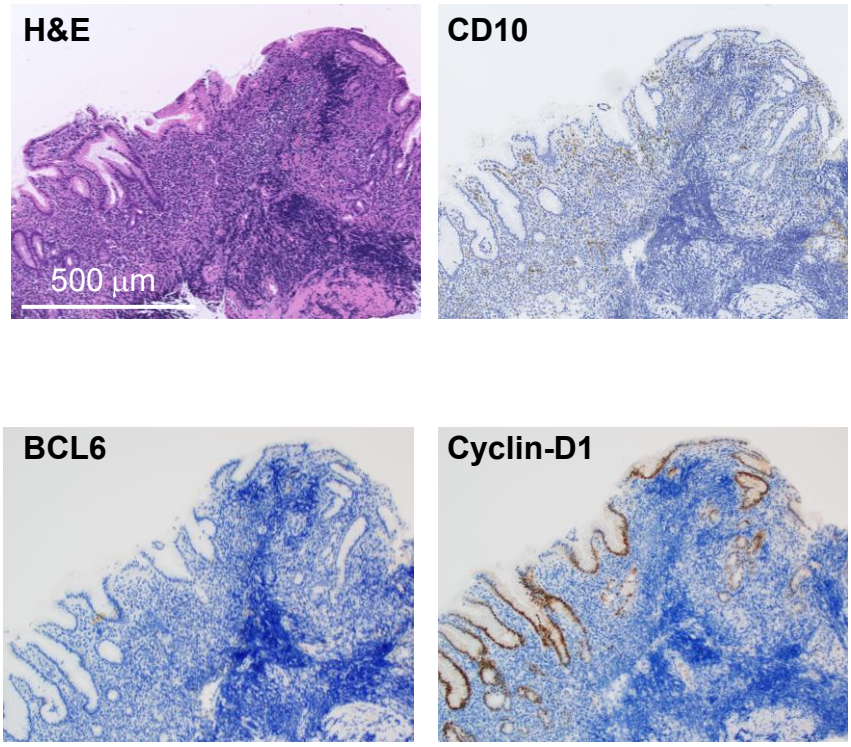

Fig. S3. Histological examination of gastric biopsies obtained from patient A.

Lymphocytes were all negative for CD10, BCL6, and Cyclin D1.

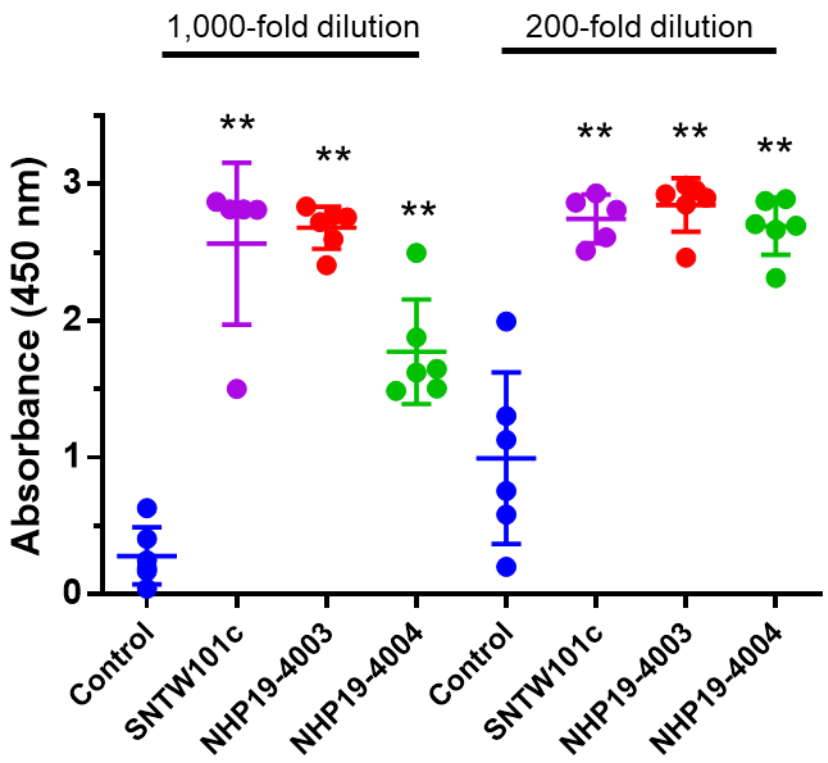

Fig. S4. *H. suis*–specific IgG levels in serum of *H. suis*–infected mice and control mice.
The bars indicate mean±S.D. Each of the *H. suis*–infected samples was compared with the control sample using one-way ANOVA with Dunnett's test. **p<0.01.

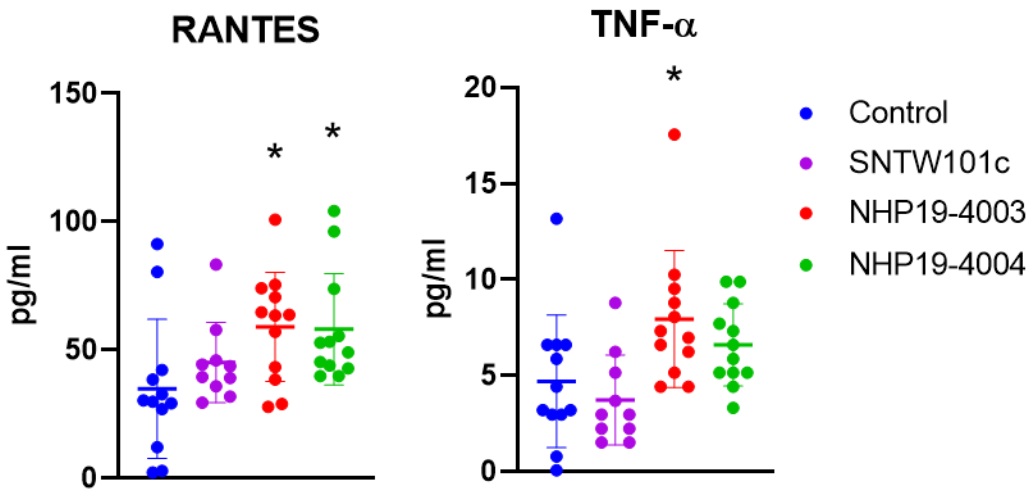

Fig. S5. Serum RANTES and TNF-α concentrations in *H. suis*–infected mice and control mice.

The bars indicate mean±S.D. Each of the *H. suis*–infected samples was compared with control samples using one way ANOVA and Dunnett's test. *p<0.05.

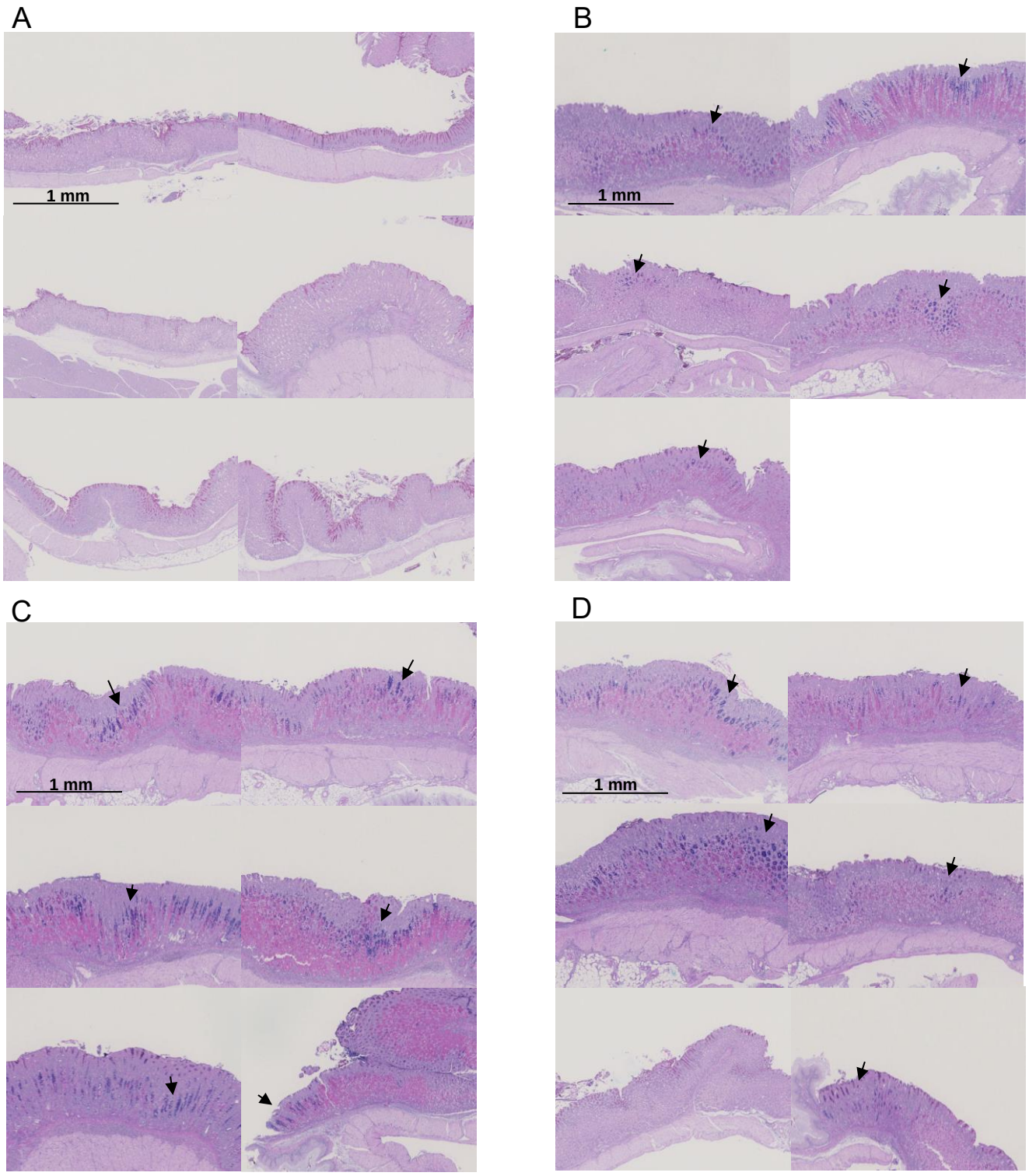

Figure S6. Alcian blue–PAS staining of gastric mucosa of *H. suis*–infected mice and control mice.

A, Control mice (n=6); B, SNTW101c-infected mice (n=5); C, NHP19-4003–infected mice (n=6); D, NHP19-4004–infected mice (n=6) . Acidic mucus, stained blue by Alcian blue–PAS staining (arrows), was observed in *H. suis*–infected mice.

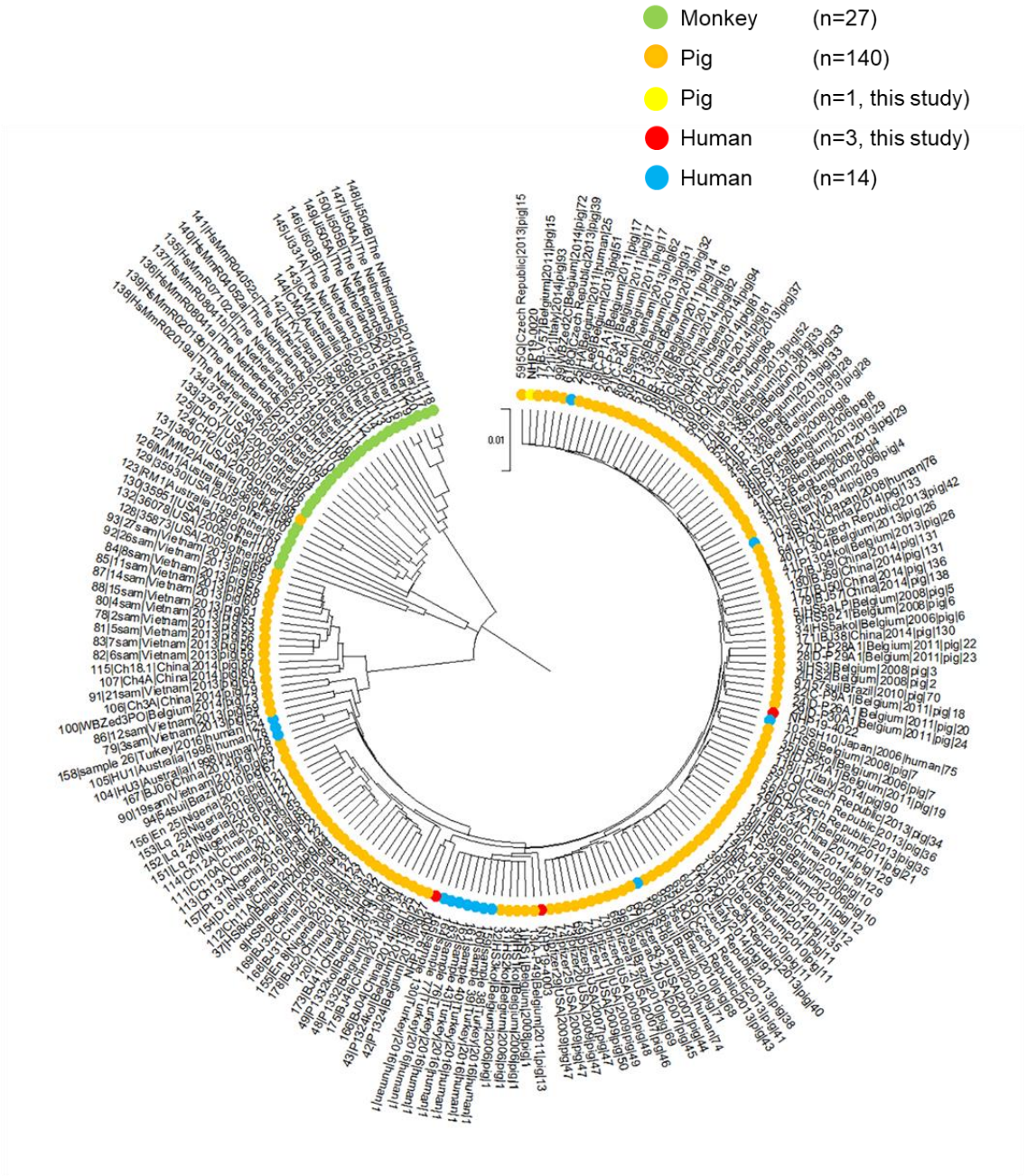

Figure S7. Phylogenetic analysis based on MLST data of *H. suis* isolates.

Phylogenetic tree using the data sets of the indicated 181 isolates from PubMLST and four isolates in from this study. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. There was a total ofThe final dataset contained 4,084 positions in the final dataset. Green, orange, yellow, red, and blue circles indicate isolates from monkeys (PubMLST), pigs (PubMLST), pigs (this study), humans (this study), and humans (PubMLST), respectively.

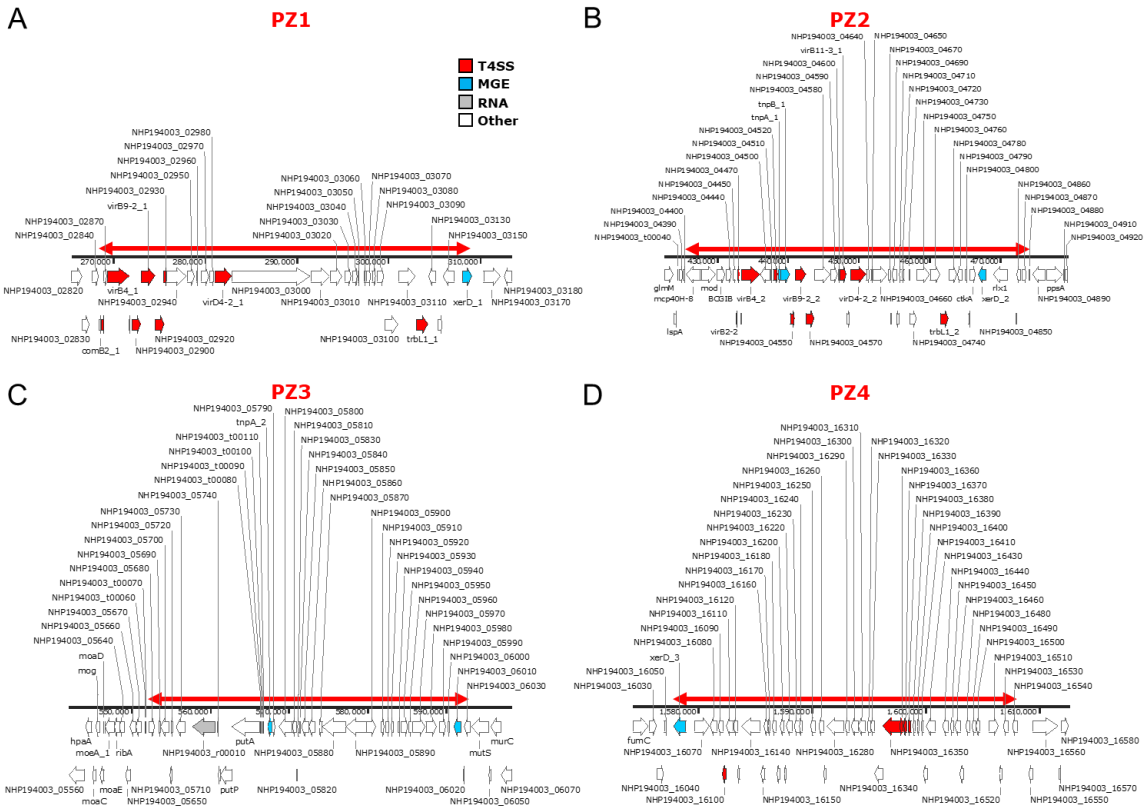

Figure S8. Plasticity zone regions in *H. suis* chromosomes.

Schematic representation of genes in plasticity zone (PZ) regions in NHP19-4003. (A) PZ1 containing genes between NHP194003_02860 and NHP194003_03160. (B) PZ2 containing genes between NHP194003_04410 and NHP194003_04880. (C) PZ3 containing genes between NHP194003_05680 and NHP194003_06030. (D) PZ4 containing genes between NHP194003_16060 and NHP194003_16540. Genes colored in red, blue, gray, and white indicate type IV secretion-associated genes (T4SS), mobile gene elements (MGE), RNA, and other genes, respectively.

Table S1. Summary of the effect of eradication of non–*Helicobacter pylori* Helicobacters in gastric disease

Authors	Year	Country	Number of cases	Median age (range)	Sex (M : F)	Diagnosis	NHPH identification (species)	Eradication regimen	Outcome
Goddard AF *et al.* (1)	1997	UK	1	47	1:0	DU	Histology	O80+DeNol480+TC2000+ M1200 / 14Td	Effective
YamamotoT *et al.* (2)	1999	Japan	1	71	1:0	EG	Histology	O20+A1500+ES / 14Td	Effective
Morgner A *et al.* (3)	2000	Germany	5	65 (42-79)	3:2	MALT	Histology and PCR[#1]	O120+A2250 / 14Td	Effective
Yoshimura M *et al.* (4)	2002	Japan	1	69	0:1	AGML	Histology, EM	L60+M500+C400 / 14Td	Effective
Kato S *et al.* (5)	2005	Japan	1	11	1:0	DU	Histology	L40+A1500+C800 / 7Td	Effective
Okiyama Y *et al.* (6)	2005	Japan	2	-	-	MALT	Histology	L60+A1500+C800 / 7Td	Effective
Siala K *et al.* (7)	2007	Czech	1	17	1:0	CG	Histology	OACM[#2]	Effective
Ohtaka M *et al.* (8)	2012	Japan	1	64	0:1	GP	Histology?	L60+A1500+C400 / 7Td	Effective
Joosten M *et al.* (9)	2013	Belgium	1	33	1:0	Dyspepsia	Histology and PCR (*H. suis*)	P80+A2000+C1000 / 10Td	Effective
Okamura I *et al.* (10)	2013	Japan	1	46	1:0	MALT	Histology and PCR (*H. suis*)	R20+A1500+C800 / 7Td	Effective
Matsumoto I *et al.* (11)	2014	Japan	1	62	0:1	GU	Histology and PCR (*H. heilmannii*)	L30A1500C1200 / 7Td	Effective
Goji S *et al.* (12)	2015	Japan	1	48	0:1	NG	Histology and PCR (*H. suis*)	E40+A1500+C / 7Td	Effective
Shiratori S *et al.* (13)	2016	Japan	1	48	1:0	CG	Histology and PCR (*H. suis*)	R20+A1500+C / 7Td	Effective
Nakagawa S *et al.* (14)	2018	Japan	1	56	1:0	MALT	Histology and PCR (*H. suis*)	E40+A1500+M500 / 10Td	Effective
Takigawa H *et al.* (15)	2019	Japan	4	40 (36-45)	4:0	MALT	Histology and PCR (*H. suis*)	L60+A1500+C400 / 7Td	Effective
Nakamura M *et al.* (16)	2020	Japan	49	-	-	Gastric disease	PCR[#3]	PPI+A+C or M / 7 or 10Td	Effective

NHPH: non–*Helicobacter pylori* Helicobacters*;* EM: electron micrograph; O: omeprazole; E: esomeprazole; L: lansoprazole; R: rabeprazole; P: pantoprazole; A: amoxicillin; C: clarithromycin; M: metronidazole; TC: tetracycline; ES: ecabet sodium; NG: nodular gastritis; CG: chronic gastritis; GU: gastric ulcer; GP: gastric plasmacytoma; AGML: acute gastric mucosal lesions; EG: erosive gastritis; MALT: gastric MALT lymphoma.

[#1] Two of five patients were identified as having *H. heilmannii* type I (has been identified as *H. suis*) by sequencing of the 16S rRNA gene.

[#2] Unknown number of days.

[#3] Twenty cases were *H. suis*, 7 were *H. heilmannii* / *Helicobacter ailurogastricus*, and the other 22 cases were unknown.

Table S2. Summary of genome sequences of *H. suis* isolates

Strain	Accession No.	Total Length	No. of Sequences	GC Content	N50	No. of CDSs	No. of RNAs	Coding Ratio
Complete genome								
NHP19-4003	AP023039-AP023041	1,715,041 bp	3 (2 plasmids)	40.00%	1,689,367	1,757	44	90.80%
NHP19-4004	AP023042-AP023045	1,738,770 bp	4 (3 plasmids)	40.00%	1,703,808	1,776	45	89.60%
NHP19-4022	AP023046-AP023048	1,682,860 bp	3 (2 plasmids)	40.10%	1,670,524	1,733	44	90.70%
NHP19-0020	AP023036-AP023038	1,777,109 bp	3 (2 plasmids)	39.80%	1,726,209	1,835	45	90.80%
SNTW101c	AP019774-AP019776	1,694,897 bp	3 (2 plasmids)	40.00%	1,680,021	1,744	44	90.70%
Draft genome								
NHP19-0033	BLRI01000000	1,662,732 bp	163	39.90%	22,958	1,604	41	86.20%
HS1	ADGY00000000	1,625,992 bp	78	39.90%	46,022	1,605	41	88.40%
HS2	FZLI00000000	1,632,057 bp	84	40.00%	44,089	1,613	42	88.30%
HS3	FZKT00000000	1,647,490 bp	81	40.00%	42,680	1,632	42	88.60%
HS4	FZKI00000000	1,639,168 bp	84	39.90%	46,278	1,609	42	88.40%
HS5	ADHO00000000	1,644,767 bp	78	39.90%	44,807	1,623	42	88.60%
HS6	FZLD00000000	1,641,870 bp	84	40.00%	44,929	1,620	42	88.50%
HS7	FZKH00000000	1,636,417 bp	82	39.90%	45,589	1,627	41	88.90%
HS8	FZKJ00000000	1,637,926 bp	89	40.00%	38,616	1,621	41	88.50%
HS9	FZLE00000000	1,644,324 bp	86	40.00%	48,752	1,619	43	88.00%
HS10	FZKV00000000	1,655,684 bp	81	39.90%	48,664	1,647	42	89.20%

The indicated data of complete genomes of isolates NHP19-4003 (human), NHP19-4004 (human), NHP19-4022 (human), NHP19-0020 (pig), and SNTW101c (human, mouse adapted) (A) and draft genomes of isolates NHP19-0033, HS1, HS2, HS3, HS4, HS5, HS6, HS7, HS8, HS9, and HS10 (all from pigs) are shown (B).

Table S3. Summary of genome sequencing analysis of *H. suis* isolates recovered from mouse stomachs 4 months after infection

Isolate	Mouse No.	Accession No.	Mapped reads	Coverage	Mapped reads	Total mutations	Non-synonymous mutations
A							
NHP19-4003_1-1	1	DRS142271	146,439,307 bp	85.39x	99.40%	25	18
NHP19-4003_1-4	1	DRS142272	163,483,997 bp	95.32x	99.46%	26	17
NHP19-4003_2-4	2	DRS142273	165,392,773 bp	96.44x	99.32%	23	16
NHP19-4003_3-1	2	DRS142274	150,763,986 bp	87.91x	99.52%	20	14
NHP19-4003_3-2	3	DRS142275	166,318,659 bp	96.98x	99.50%	30	15
NHP19-4003_3-3	3	DRS142276	183,896,780 bp	107.23x	99.42%	21	13
NHP19-4003_4-3	4	DRS142277	159,051,676 bp	92.74x	99.44%	24	15
NHP19-4003_4-4	4	DRS142278	185,469,156 bp	108.14x	99.43%	22	16
NHP19-4003_5-1	5	DRS142279	179,975,089 bp	104.94x	99.48%	23	13
NHP19-4003_5-2	5	DRS142280	172,565,948 bp	100.62x	99.42%	21	13
NHP19-4003_6-1	6	DRS142281	168,419,994 bp	98.20x	99.42%	17	13
NHP19-4003_6-3	6	DRS142282	179,574,855 bp	104.71x	99.46%	20	15
B							
NHP19-4004_1-1	1	DRS142283	229,711,303 bp	132.11x	98.12%	12	5
NHP19-4004_1-2	1	DRS142284	229,394,812 bp	131.93x	97.45%	16	9
NHP19-4004_2-1	2	DRS142285	233,314,312 bp	134.18x	97.24%	13	7
NHP19-4004_2-2	2	DRS142286	219,196,450 bp	126.06x	97.20%	15	8
NHP19-4004_3-1	3	DRS142287	162,880,464 bp	93.68x	97.04%	11	4
NHP19-4004_3-2	3	DRS142288	153,877,060 bp	88.50x	97.32%	11	5
NHP19-4004_4-1	4	DRS142289	102,541,596 bp	58.97x	98.25%	11	6
NHP19-4004_4-2	4	DRS142290	139,688,786 bp	80.34x	96.33%	11	6
NHP19-4004_5-1	5	DRS142291	145,116,103 bp	83.46x	97.52%	9	4
NHP19-4004_5-2	5	DRS142292	165,921,687 bp	95.42x	98.15%	9	4
NHP19-4004_6-1	6	DRS142293	156,507,638 bp	90.01x	96.58%	10	4
NHP19-4004_6-2	6	DRS142294	378,168,261 bp	217.49x	96.63%	20	9

The indicated data from reference mapping of sequencing reads of 12 isolates from NHP19-4003–infected mice to the NHP19-4003 genome (A) and reference mapping of sequencing reads of 12 isolates from NHP19-4004–infected mice to the NHP19-4004 genome are shown (B).

Table S4. Definitions for histopathological scoring of mouse stomach sections

Score	Density of neutrophils in the gastric muscularis mucosa	Lymphocytic infiltration in the gastric muscularis mucosa	Degree of mucosal metaplasia	Lymph follicles
0	None	None	None	None
1	Up to 50% of crypts (area) in the section involved	10 cells /high-power field	One focal area (one to four crypts) in a section	Number of lymph follicles (>200 μm) are shown.
2	More than 50% of crypts (area) in the section involved	Some areas with dense lymphocytes	Multiple foci in a section	
3	All crypts (area) in the section involved	Diffuse infiltration with dense lymphocytes	More than 50% gastric epithelium diffusely replaced by mucosal metaplasia	

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