Isolation and characterization of *Helicobacter suis* from human stomach

Emiko Rimbara[a,1,2], Masato Suzuki[b,1], Hidenori Matsui[c,2], Masahiko Nakamura[d], Misako Morimoto[e], Chihiro Sasakawa[e,f], Hiroki Masuda[g,h], Sachiyo Nomura[g], Takako Osaki[i], Noriyo Nagata[j], Keigo Shibayama[a], and Kengo Tokunaga[k,2]

[a]Department of Bacteriology II, National Institute of Infectious Diseases, 208-0011 Tokyo, Japan; [b]Antimicrobial Research Center, National Institute of Infectious Diseases, 189-0002 Tokyo, Japan; [c]Omura Satoshi Memorial Institute, Kitasato University, 108-8641 Tokyo, Japan; [d]School of Pharmacy, Kitasato University, 108-8641 Tokyo, Japan; [e]Department of Research Associate Product Development, Nippon Institute for Biological Science, 198-0024 Tokyo, Japan; [f]Medical Mycology Research Center, Chiba University, 263-8522 Chiba, Japan; [g]Department of Gastrointestinal Surgery, Graduate School of Medicine, The University of Tokyo, 113-8654 Tokyo, Japan; [h]Department of Gastrointestinal Surgery, Nippon Medical University, 113-8602 Tokyo, Japan; [i]Department of Infectious Diseases, Kyorin University School of Medicine, 181-8611 Tokyo, Japan; [j]Department of Pathology, National Institute of Infectious Diseases, 208-0011 Tokyo, Japan; and [k]Department of General Medicine, Kyorin University School of Medicine, 181-8611 Tokyo, Japan

Helicobacter suis, a bacterial species naturally hosted by pigs, can colonize the human stomach in the context of gastric diseases such as gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Because *H. suis* has been successfully isolated from pigs, but not from humans, evidence linking human *H. suis* infection to gastric diseases has remained incomplete. In this study, we successfully in vitro cultured *H. suis* directly from human stomachs. Unlike *Helicobacter pylori*, the viability of *H. suis* decreases significantly on neutral pH; therefore, we achieved this using a low-pH medium for transport of gastric biopsies. Ultimately, we isolated *H. suis* from three patients with gastric diseases, including gastric MALT lymphoma. Successful eradication of *H. suis* yielded significant improvements in endoscopic and histopathological findings. Oral infection of mice with *H. suis* clinical isolates elicited gastric and systemic inflammatory responses; in addition, progression of gastric mucosal metaplasia was observed 4 mo postinfection. Because *H. suis* could be isolated from the stomachs of infected mice, our findings satisfied Koch's postulates. Although further prospective clinical studies are needed, *H. suis*, like *H. pylori*, is likely a gastric pathogen in humans. Furthermore, comparative genomic analysis of *H. suis* using complete genomes of clinical isolates revealed that the genome of each *H. suis* isolate contained highly plastic genomic regions encoding putative strain-specific virulence factors, including type IV secretion system–associated genes, and that *H. suis* isolates from humans and pigs were genetically very similar, suggesting possible pig-to-human transmission.

Helicobacter suis | gastric diseases | zoonosis

Non-*Helicobacter pylori* Helicobacters (NHPH), which have a corkscrew-like spiral morphology very different from that of *H. pylori*, have been known to be present in human stomachs since the 1980s (1). NHPH infection has been observed in patients with gastric diseases, including peptic ulcers, chronic gastritis, gastric cancers, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (2–4).

Because routine diagnostic methods based on the urease activity of *H. pylori*, such as the urea breath test and rapid urease test, often yield negative results for NHPH (5), and the bacteria is uncultivable, NHPH infection in individual patients could be missed. Thus, in the post-*H. pylori* era, NHPH could become an important gastric pathogen in people of all ages.

The primary human pathogenic NHPH species is *Helicobacter suis*, which is naturally hosted by pigs (6). *H. suis* infects 60 to 95% of livestock pigs, in which it causes gastritis, ulcers, and a reduced rate of weight gain (6, 7). Humans are frequently infected by *H. pylori* in childhood but rarely in adulthood (8); by contrast, the route and period of *H. suis* infection in humans is completely unknown. In some cases, *H. suis* was observed in patients with gastric diseases after *H. pylori* eradication therapy

(9). It is not known whether only *H. pylori* was eradicated in individuals coinfected by *H. pylori* and *H. suis* or if instead *H. suis* arose after *H. pylori* eradication.

Successful isolation and in vitro culture of *H. pylori* has dramatically contributed to elucidating the causal relationships between bacterial infection and gastric diseases as well as the molecular and cellular mechanisms of gastric cancer pathogenesis (10, 11). Successful isolation and in vitro culture of *H. suis* from pig stomach were reported in 2008 (12), and animal infection experiments using *H. suis* isolates from pigs demonstrated its pathogenicity. In addition, previous reports strongly suggested that *H. suis* infection contributes to gastric diseases in humans (*SI Appendix*, Table S1). However, studies on *H. suis* have been hindered because *H. suis* has not been successfully isolated from humans. In other words, the first and third of Koch's postulates (13) ("association of the microbe with symptoms and presence of the microbe at the site of infection" and "reproduction of the disease by inoculation of a susceptible host with the microbe") have been partially achieved for *H. suis*, whereas

Significance

This study directly demonstrates virulence-associated features of *Helicobacter suis* infection using isolates obtained from human stomachs. *H. suis* is the second-most-prevalent *Helicobacter* species in the human stomach, but diagnostic tests for *Helicobacter pylori* often fail to diagnose *H. suis*. *H. suis* does not have orthologs of either cytotoxin-associated gene A (CagA) or vacuolating cytotoxin A (VacA), two major virulence factors in *H. pylori*, suggesting that the molecular pathogenicity of *H. suis* and clinical manifestations caused by *H. suis* infection are different from those caused by *H. pylori*. This study paves the way for epidemiological research aimed at identifying the causal relationships between *H. suis* and gastric diseases and developing diagnostic methods.

the remaining two postulates ("isolation of the microbe from lesions" and "re-isolation of the microbe from experimentally infected individuals") have not.

We developed a modified culture method for a human isolate, *H. suis* strain SNTW101c (14), which was isolated in 2008 from a patient with nodular gastritis and has been passaged in mouse stomach for 12 y by repeated inoculations of uninfected mice with gastric mucosal homogenates from infected mice (15). In this study, we succeeded in culturing *H. suis* directly from human gastric biopsies and demonstrated *H. suis* virulence in humans based on Koch's postulates using an experimental animal model of *H. suis* infection. We also determined the complete genomes of *H. suis* isolates from humans and pigs and compared their genetic features.

Results

Isolation and Characterizations of *H. suis* from Humans and Pigs. *H. suis* isolates were successfully cultured in vitro from patients A, B, and C, and named NHP19-4003, NHP19-4004, and NHP19-4022, respectively. *H. suis* isolates from slaughter pigs in Japan were named NHP19-0020 and NHP19-0033. In all cases, small clear colonies of *H. suis* on NHPH agar plates, smaller than those formed by *H. pylori*, appeared 8 to 10 d after inoculation, as shown in *SI Appendix*, Fig. S1. Scanning electron microscopy of *H. suis* isolates revealed a corkscrew-like spiral morphology with long bipolar flagella, which contribute to the motility of this bacterium (Fig. 1, https://www.youtube.com/watch?v=sHA4SSzu4eo&feature=youtu.be). Urease activities were detected in all isolates, although the rapid urease test and urea breath test were negative in all patients, in concordance with a previous report (*SI Appendix*, Table S1). All isolates were identified as *H. suis* by comparing average nucleotide identity (ANI) of whole-genome sequences of the isolates with *H. suis* strain HS5, the reference strain, which was originally isolated from a pig (accession no. NZ_ADHO01000000). ANI values of NHP19-4003, NHP4004, NHP19-4022, and NHP19-0020 were 99.64, 99.64, 99.74, and 99.64%, respectively.

In regard to antimicrobial susceptibility, none of the four *H. suis* isolates obtained in this study, as well as *H. suis* strain SNTW101c, were resistant to clarithromycin, levofloxacin, and oxacillin (Table 1). Minimum inhibitory concentrations (MICs) of amoxicillin in four of five isolates were 1.0 mg/L but were higher (16 mg/L) in one isolate, NHP19-4004. In that isolate, comparison of amino acid sequences of PBPA (PBP1), PBP2, and FtsI (PBP3), which are penicillin-binding proteins (PBPs) targeted by β-lactams, revealed the presence of one and three substitutions in the transpeptidase region (target region of β-lactams) in

Table 1. Antimicrobial susceptibility of the *H. suis* strain SNTW101c and *H. suis* strains obtained in this study

Isolates	Origin	MIC (mg/L) of:						
		AMPC	OX	CAM	MINO	GM	LVFX	MTZ
SNTW101c	Human*	1	≤0.25	≤0.25	≤2	≤4	≤0.5	16
NHP19-4003	Human	1	≤0.25	0.5	≤2	≤4	≤0.5	8
NHP19-4004	Human	≥16	≤0.25	0.5	8	≤4	1	16
NHP19-4022	Human	1	≤0.25	≤0.25	≤2	≤4	≤0.5	16
NHP19-0020	Pig	1	≤0.25	≤0.25	4	≤4	≤0.5	8

AMPC: Amoxicillin; OX: Oxacillin; CAM: Clarithromycin; MINO: Minocycline; GM: Gentamicin; LVFX: Levofloxacin; MTZ: Metronidazole.
*Mouse-adapted.

PBP2 and FtsI, respectively. The substitution at position 317 in PBP2 and the two substitutions at positions 532 and 547 were sterically close to the penicillin-binding motif in the predicted three-dimensional structures of PBPs (*SI Appendix*, Fig. S2). No specific substitutions were observed in the transpeptidase region of PBPA.

Effects of *H. suis* Eradication on Gastric Diseases in Patients. Endoscopic and histological data of patients pre- and posteradication were available for patient A and patient B.

In patient A, thickened mucosae and nodular irregularities in the lesser curvature of the angle were observed endoscopically (Fig. 2*A*). Lymphocyte infiltration and formation of lymphoepithelial lesions were observed in biopsy tissues by hematoxylin–eosin (H&E) staining (Fig. 2*B*). Lymphocytes were CD20 positive but negative for CD3 (Fig. 2*B*), CD10, BCL6, and Cyclin D1 (*SI Appendix*, Fig. S3), as determined by immunohistochemistry. Patient A was diagnosed with *H. suis*–infected gastric MALT lymphoma with nodular gastritis and treated with the standard triple therapy for *H. pylori*: 750 mg amoxicillin, 400 mg clarithromycin, and 20 mg vonoprazan twice daily for 7 d. Successful eradication was confirmed by both PCR and culture 3 mo later. The gastric MALT lymphoma was confirmed to be in complete remission 9 mo later (Fig. 2 *A* and *B*).

Patient B was diagnosed with multiple *H. suis*–infected gastric ulcers by endoscopy (Fig. 2*A*) and histology (Fig. 2*C*) and was successfully treated with the same regimen as patient A (Fig. 2 *A* and *C*). Long and tightly coiled spiral microorganisms, distinct from *H. pylori*, were observed by Giemsa staining in patients A and B (Fig. 2 *B* and *C*).

Patient C was diagnosed with nodular gastritis and successfully treated with the same regimen as patient A.

Effects of *H. suis* Infections in Mice. Clinical isolates of *H. suis* NHP19-4003 and NHP19-4004 and the mouse-adapted strain SNTW101c were used for experimental infection of mice. Because we knew that at least 3 mo were required to see the pathogenetic features in previous mouse infection experiments using the mouse-adapted strain TKY (16), we selected an infection duration of 4 mo for this study.

All isolates successfully infected the mice. The 4 mo after inoculation isolates were recovered from all mice. Comparison of bacterial numbers between isolates revealed that SNTW101c-infected mice contained 16 and 13 times as many bacteria as NHP19-4003– and NHP4004–infected mice, respectively (Fig. 3*A*). *H. suis* infection was further confirmed by serum *H. suis*–specific IgG measured by enzyme-linked immunosorbent assay (*SI Appendix*, Fig. S4).

RANTES and TNF-α levels were both significantly higher in NHP19-4003–infected mice than in control mice, and RANTES level was significantly higher in NHP19-4004–infected mice than in control mice (*SI Appendix*, Fig. S5).

H&E staining of gastric mucosal tissue revealed lymphocyte infiltration in stomach mucosa of all *H. suis*–infected mice
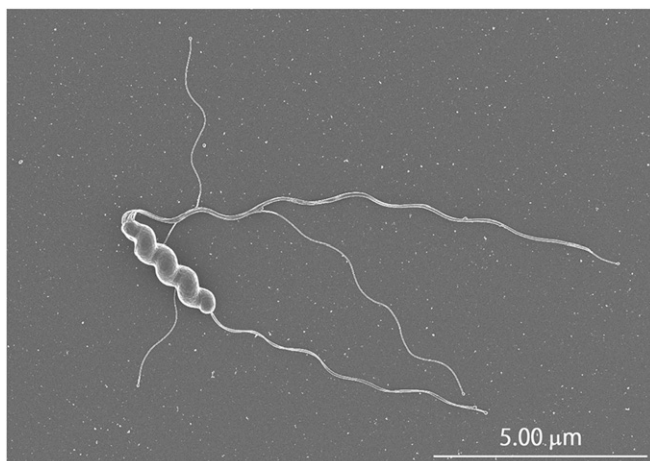

Fig. 1. Scanning electron micrograph of *H. suis* strain NHP19-4003 from a human patient.
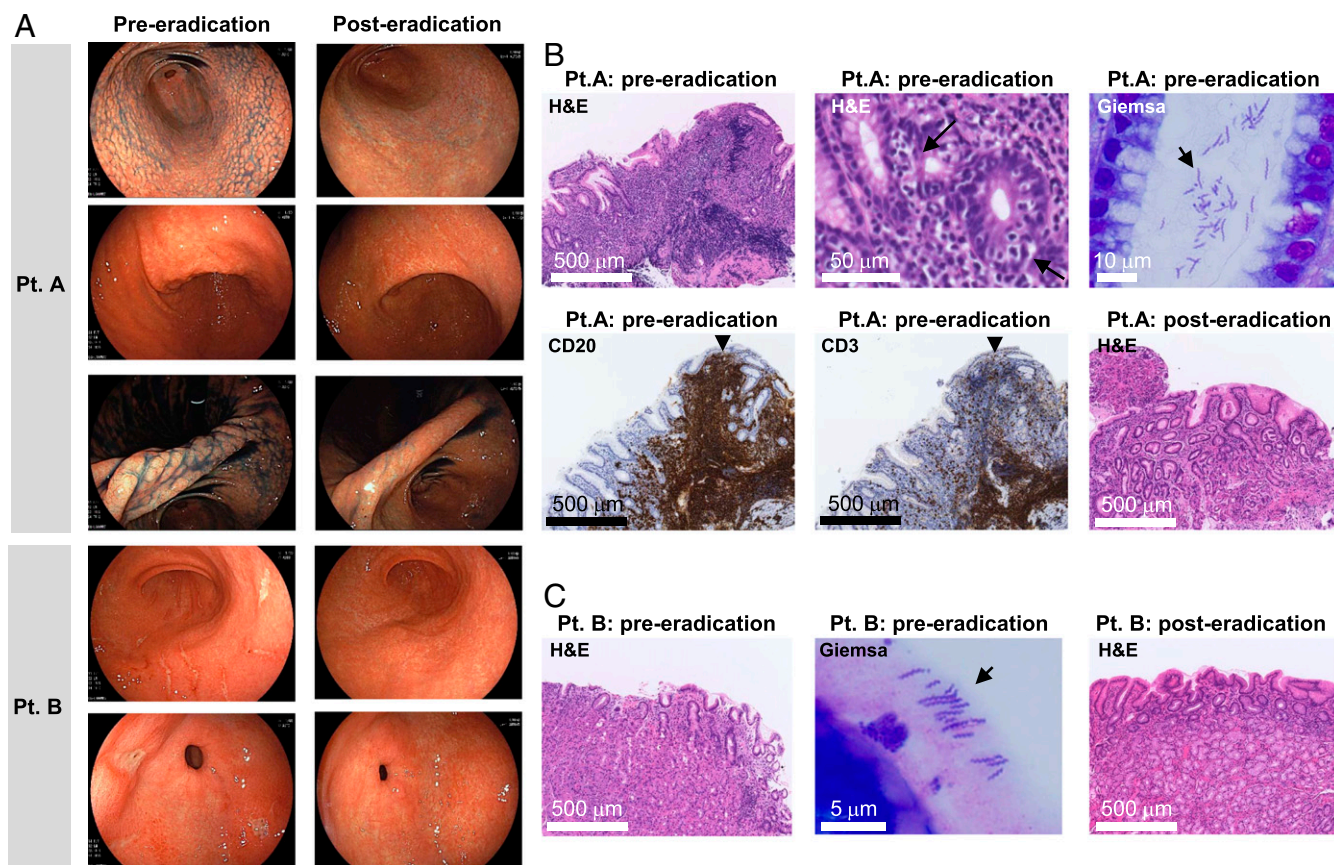
Fig. 2. Endoscopic and histological images from *H. suis*–infected patients. (*A*) Endoscopic images obtained from patient A and patient B pre- and poster-adication. Patient A: Nodular gastritis from the antrum to the angle and the mucosal thickening in the angle of the stomach, endoscopically observed preeradication, improved 9 mo after eradication. Patient B: The open and linear multiple gastric ulcers observed before eradication disappeared. Histological examination of gastric biopsies obtained from patient A (*B*) and patient B (*C*) pre- and posteradication. Patient A: Preeradication, diffuse infiltration of lymphocytes and lymphoepithelial lesion (arrow) were observed by H&E staining. Immunostaining of infiltrating lymphocytes within the follicle was positive for CD20 and negative for CD3. Improvement of diffuse infiltration of lymphocytes and improvement of lymphoepithelial lesions were confirmed 3 mo after eradication. Patient B: Improvement of an infiltration of neutrophils observed by H&E staining pretreatment was confirmed posteradication. In both patients, bacteria with spiral morphology very different from that of *H. pylori* were observed by Giemsa staining.

(Fig. 3 *D* and *E*). Significant neutrophil infiltration was observed in muscularis mucosae and/or mucosa of stomach in some NHP19-4003– and NHP19-4004–infected mice (Fig. 3 *D* and *E*). Lymphoid aggregates were observed in most *H. suis*–infected mice, and lymphoid follicles were especially prominent in SNTW101c-infected mice (Fig. 3 *B* and *C*). Alcian blue– periodic acid-Schiff (PAS) staining revealed mucosal metaplasia in almost all *H. suis*–infected mice (Fig. 3 *D* and *E* and *SI Appendix*, Fig. S6). Mucosal metaplasia was further evaluated by staining for the trefoil factor 2 (TFF2) and trefoil factor 3 (TFF3), representing spasmolytic polypeptide-expressing metaplasia (SPEM) and intestinal metaplasia (IM), respectively. Most *H. suis*–infected mice were positive for TFF2 and TFF3 and were confirmed to have late-stage SPEM or IM. TFF2 was coexpressed with TFF3 in most stomach glands of *H. suis*–infected mice (Fig. 3 *D* and *G*). The percentage of glands expressing TFF2 or TFF3 was calculated in three randomly selected mice in each group, revealing significant expression of TFF2 and TFF3 in all NHP19-4003–infected mice and two of three SNTW101c- and NHP19-4004–infected mice. By contrast, none of the control mice expressed TFF2 or TFF3 (Fig. 3*F*).

Genomic Comparisons of *H. suis* between Isolates from Humans and Pigs. Genome sequences of *H. suis* isolates from humans and pigs (NHP19-4003, NHP19-4004, and NHP19-4022 from humans; and

NHP19-0020 and NHP19-0033 from pigs) were determined by short- and long-read sequencing (*SI Appendix*, Table S2). The complete genomes of four isolates (NHP19-4003, NHP19-4004, NHP19-4022, and NHP19-0020) and the draft genome of NHPH190033 were determined. The complete genomes of the four isolates revealed that *H. suis* harbors plasmids carrying genes associated with DNA restriction–modification systems, as previously reported in SNTW101c (14). Importantly, *H. suis* was recovered from all stomachs of infected mice, and short-read sequencing revealed that their genomes were identical to those of the in-oculated isolates (*SI Appendix*, Table S3), suggesting that *H. suis* infection was involved in the observed symptoms.

Based on multilocus sequence typing (MLST) analysis, NHP19-4003, NHP19-4004, and NHP19-4022 belonged to sequence types (STs), which were assigned as ST139, ST140, and ST141, respectively. Both NHP19-0020 and NHP19-0033 belonged to ST15, like the pig isolates B-V57 and 5Q from Belgium and Czech Republic, respectively. Phylogenetic trees constructed from a set of MLST data of 185 isolates, including five isolates in this study, revealed that all human isolates were scattered among the branches of pig isolates, suggesting that human and pig isolates are genetically very close (*SI Appendix*, Fig. S7).

Genome-wide characterization and phylogenetic analysis of 16 genomes of *H. suis*, including 5 isolates from this study and 10 pig isolates from Belgium (17), further suggested a high genomic
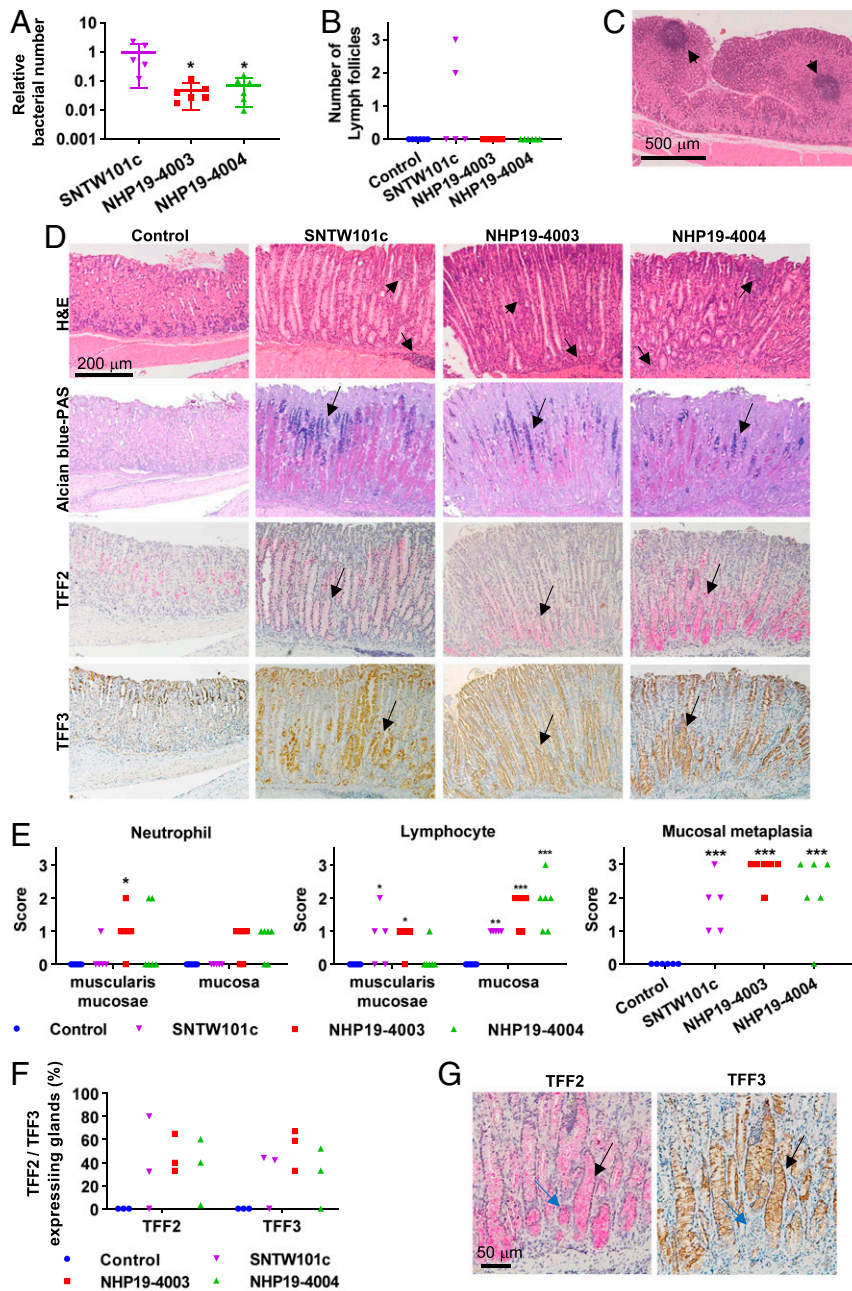
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Fig. 3. Effects of *H. suis* infection in mice. (*A*) Relative bacterial number of *H. suis* in the mouse stomach. Bars indicate means ± SD. (*B*) Number of lymph follicles observed in stomach sections from *H. suis*–infected and control mice. (*C*) H&E staining of a lymph follicle (arrows) observed in mice infected with *H. suis* SNTW101c. (*D*) H&E, Alcian blue–PAS, TFF2, and TFF3 staining of stomach sections from *H. suis*–infected and control mice. Lymphocyte infiltration in mucosa and muscularis mucosae (arrows) was observed in *H. suis*–infected mice. Acidic mucus, stained blue with Alcian blue–PAS (arrows), was observed in *H. suis*–infected mice. Expression of TFF2 and TFF3 was observed at the bases of glands (arrows) in *H. suis*–infected mice. (*E*) Scoring of neutrophil and lymphocyte infiltration in muscularis mucosae and mucosa and mucosal metaplasia of stomach sections from *H. suis*–infected and control mice. Bars indicate means ± SD. (*F*) Percentage of glands expressing TFF2 or TFF3 in *H. suis*–infected and control mice. (*G*) TFF2- or TFF3-expressing glands of stomach from *H. suis* NHP19-4004–infected mice. Most glands expressed both TFF2 and TFF3 (black arrows), and rare glands (blue arrows) expressed only TFF2. In each of the *H. suis*–infected samples, the relative bacterial number of *H. suis* in the stomach of NHP19-4003– or NHP19-4004–infected mice was compared with that of SNTW101c–infected mice by one-way ANOVA with Dunnett's test. Scores for cell infiltration observed in stomach sections in *H. suis*–infected mice were compared with those in control mice by two-way ANOVA with Sidak's multiple comparisons test. Numbers of lymph follicles and scores for mucosal metaplasia in *H. suis*–infected mice were compared with those in controls by one-way ANOVA with Holm–Sidak's multiple comparisons test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

similarity between isolates from humans and pigs (Fig. 4*A*). Total numbers of coding sequences of *H. suis* were from 1,604 to 1,835 (the mean was 1,667). *H. suis* isolates shared a core genome of 1,306 genes, and therefore, each isolate possessed an accessory genome consisting of several hundred strain-specific genes, which resulted in a 2,392-gene pangenome (Fig. 4*A*). Comparative genomic

analysis of five chromosomes of *H. suis* isolates (NHP19-4003, NHP19-4004, NHP19-4022, NHP19-0020, and SNTW101c) revealed that large recombination events have occurred in these genomes (Fig. 4*B*). Of note, each chromosome contained four nonconserved genomic regions, named plasticity zones (PZs) 1 to 4, which often contained genes associated with the type IV secretion system (T4SS),

such as T4SS-specific ATPase genes *virB4*, *virB11*, and *virD4*, as in the *cag* pathogenicity island (*cag*PAI) in *H. pylori* (Fig. 4*B* and *SI Appendix*, Fig. S8).

Discussion

NHPH in the human stomach was named "*Helicobacter heilmannii*" in acknowledgment of Konrad Heilmann's work. In the 1990s, DNA sequencing of the 16S ribosomal RNA (rRNA) genes of NHPH from humans revealed that "*H. heilmannii*" included several species; however, the identification of individual species was difficult because they are genetically similar and unculturable. Later, *H. suis* was shown to be the most prevalent NHPH species in the human stomach (6). *H. heilmannii* is now identified as a species from cats (18). Culture of *H. suis* and *H. heilmannii* (from pig and cat, respectively) revealed that low pH is an important condition for culture in vitro (12, 18). Our preliminary results suggested that neutral pH has a significant effect on the viability of *H. suis* even after 1 h (https://www.youtube.com/watch?v=J68oZADtDj4&feature=youtu). Therefore, we used low-pH medium for the transport of gastric biopsies in this study, enabling us to isolate *H. suis* directly from human gastric biopsies.

Successful culture of *H. suis* from humans could help to answer many questions about the best therapy for the eradication of *H. suis*, the transmission route of *H. suis*, and the virulence factors contributing to *H. suis* pathogenicity in humans. *H. suis* can be effectively eradicated using a standard triple therapy, including amoxicillin and clarithromycin, that has also been used for the eradication of *H. pylori* infection (19, 20). Although
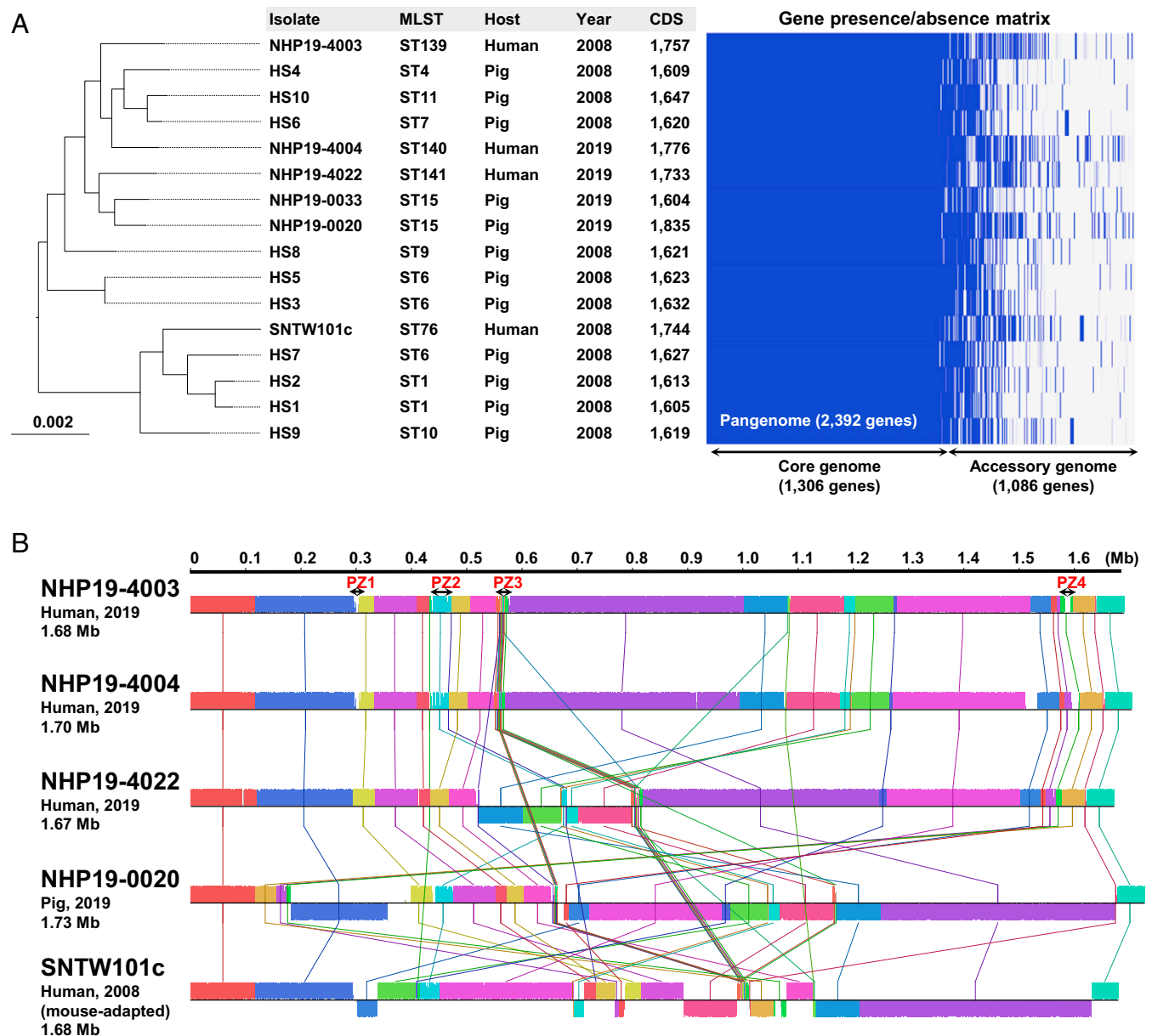

Fig. 4. Comparative analysis based on whole-genome data of *H. suis* isolates. (*A*) Pangenome analysis using genomes from the indicated 16 isolates. Phylogenetic trees generated using core genome alignments. Sequence type (ST), host, year of isolation, number of coding sequences, and gene presence/absence matrix are shown. Bar lengths represent the number of substitutions per site in the core genome. The pangenome of *H. suis* consisted of 2,392 genes, including 1,306 core genes (≥99.0% conserved in the isolates) and 1,086 accessory genes (<99.0% conserved in the isolates). (*B*) Linear sequence comparison of chromosomes of five isolates. Four genomic regions corresponding to PZs in NHP19-4003 are shown.

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clarithromycin resistance in *H. pylori* is the cause of most eradication failures in Japan (21), MICs of *H. suis* isolates obtained from humans demonstrated that three of the isolates were not resistant to clarithromycin. This is further supported by the fact that there are no mutations in the gene encoding 23S rRNA, the target protein of macrolides, corresponding to the position conferring clarithromycin resistance in *H. pylori* (22). Because low pH is necessary for *H. suis* growth, MICs of antimicrobial agents in *H. suis* tend to be higher than those in *H. pylori*, especially in the case of antimicrobial agents that degrade under acidic conditions. In this study, the MIC of amoxicillin in NHP19-4004 was high, possibly due to several mutations in PBP2 and FtsI. However, because the MIC of oxacillin in this strain was below 0.25 mg/L, these mutations are not critical for β-lactam resistance. Further studies are necessary to confirm a breakpoint for *H. suis* eradication therapy.

Comparative genomics of *H. suis* isolates from humans and pigs demonstrated that human isolates have a high genomic similarity to pig isolates (Fig. 4). *H. suis* has also been reported to infect monkeys (6, 23). A comparison of MLST data indicated that *H. suis* isolates from humans are less similar to isolates from monkeys than to isolates from pigs (*SI Appendix*, Fig. S7). Infection with *Helicobacter* spp. in the stomachs of slaughter pigs is highly prevalent (60 to 95%) in many countries (6, 7). In addition, *H. suis* infection has not been reported in animals other than pigs and monkeys (6). The *H. suis*–infected patients in this study were working in urban areas in Japan and had no direct contact with pigs or monkeys. Therefore, one possible route of infection is foodborne transmission, such as contaminated pig meat. Further analysis of *H. suis* from both pigs and humans will be necessary to identify the route of infection.

Infection of mice with *H. suis* clinical isolates NHP19-4003 and NHP19-4004, as well as the mouse-adapted strain SNYW101c, caused severe inflammatory responses in the gastric mucosa without exception. Mucosal metaplasia (SPEM and IM) characterized by the expression of TFF2 and TFF3, respectively, was observed in the stomachs of almost all *H. suis*–infected mice. Gastric IM is a preneoplastic gastric lesion that is also caused by the chronic infection of *H. pylori* in humans (11). Gastric SPEM is another type of TFF2-expressing metaplasia that is proposed to arise in chronic *H. pylori* infection in gerbils and has been detected in preneoplastic lesions in both humans and rodents (24, 25). SPEM has also been observed in *H. pylori*–infected mice, whereas IM has rarely been observed within 1 y of infection (25, 26). Observation of *H. pylori* infection in Mongolian gerbils suggests that SPEM likely represents a precursor of IM (24). In this study, we observed significant IM as well as SPEM in *H. suis*–infected mice, indicating that IM characterized by TFF3 expression in *H. suis*–infected mice may progress earlier than in *H. pylori*–infected mice. Although the effects of *Helicobacter* infection in rodents cannot be simply generalized to the effects in humans, as *H. suis* is the second-most prevalent *Helicobacter* species in human stomach, further investigations are needed to confirm the relationship between *H. suis* infection and gastric preneoplasia in human. In the case of *H. pylori*, two major virulence factors, cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA), contribute significantly to pathogenesis. CagA, the only protein secreted via the T4SS encoded in *cag*PAI in *H. pylori*, elicits gastric epithelial proliferative and inflammatory responses (11). VacA is a toxin secreted via the autotransporter system in *H. pylori* and induces gastric epithelial cell death and T cell dysfunction (11). Although *H. suis* infection in mice was associated with gastric mucosal metaplasia even earlier than in *H. pylori* infection, *H. suis* does not possess orthologs of these major virulence factors of *H. pylori*, suggesting the contribution of other virulence factors common to *H. suis* and *H. pylori*. Common pathogenic factors, so-called pathogen-associated molecular patterns (PAMPs), include bacterial components such as lipopolysaccharide, peptidoglycan, and flagellins. *H. pylori* has three additional VacA paralogs, ImaA,

VlpC, and FaaA, all of which are important for bacterial virulence; they are thought to have distinct functions (27). *H. suis* has a homolog of *H. pylori* VacA paralog-like autotransporter protein (gene product of NHP194003_11930 in NHP19-4003), which represents a candidate virulence factor. γ-glutamyl transpeptidase (GGT) is a membrane-associated enzyme that converts glutamine into glutamate and ammonia, as well as glutathione into glutamate and cysteinylglycin, and is involved in bacterial metabolism, gastric epithelial cell death, and T cell dysfunction during *H. pylori* infection (28). *H. suis* also has GGT, a potential virulence factor (29).

Nakamura et al. reported the formation of gastric MALT lymphoma in mice 6 mo after exposure to gastric mucus homogenate from mice infected with *H. suis* obtained from a cynomolgus monkey (23). In this study, infection of mice with a mouse-adapted strain of *H. suis*, SNTW101c, yielded a higher number of lymphoid follicles in the gastric mucosa than NHP19-4003 and NHP19-4004 infection (Fig. 3*B*). Bacterial load in the stomachs of SNTW101c-infected mice was more than 10-fold higher than those of NHP19-4003– and NHP19-4004–infected mice (Fig. 3*A*). Therefore, the differences in virulence-associated factors and PAMPs among *H. suis* strains could make a difference in the adaptive ability. We also observed differences in the progression of gastric mucosal metaplasia among mice infected with different *H. suis* strains: 4 mo after infection, gastric mucosal metaplasia was observed in all NHP19-4003–infected mice but not in all NHP19-4004–infected mice, even though bacterial loads did not differ (Fig. 3), suggesting that virulence differs among *H. suis* clinical isolates. Pangenomic comparison between *H. suis* isolates revealed that NHP19-4003 has 451 strain-specific genes in the accessory genome (Fig. 4*A* and https://dx.doi.org/10.17632/fvbvgz39f4.1). Although the function of most accessory genes is unknown, many T4SS-associated genes are included (*SI Appendix*, Fig. S8). Some types of *H. pylori* isolates have other T4SSs, termed *tfs3* and *tfs4*, encoded in the PZs in their chromosomes independent of *cag*PAI (30, 31). The roles of *tfs3* and *tfs4* in *H. pylori* infection remain unknown, but some studies have suggested that the additional T4SSs are associated with bacterial virulence (30, 31). *H. pylori cag*PAI T4SS spontaneously secretes cytosolic metabolites, such as peptidoglycan and heptose 1,7-bisphosphate, that are recognized as PAMPs by host innate immune systems (32). Given that T4SS in the PZ is a strain-specific virulence factor for *H. suis*, and that activity is involved in the secretion of known and unknown PAMPs, leading to inflammatory responses, T4SS might affect the risks and outcomes of infection, such as gastric IM. Further analyses, including transcriptomics, proteomics, and metabolomics, will be required to identify the functions and impacts of accessory genes encoded in PZs during *H. suis* infection.

In conclusion, we successfully isolated *H. suis* from patients with gastric diseases, reconstituted bacterial pathogenicity using clinical isolates in laboratory mice, and recovered bacteria from the infected mice. Thus, we have certified the virulence of *H. suis* as a human gastric pathogen in accordance with Koch's postulates. Further genomic epidemiological research on isolates from humans and animals will be useful for exploring the virulence of *H. suis*, its transmission to humans, and the diagnosis and treatment of *H. suis*–elicited gastric diseases. In any case, further research is needed to clarify how *H. suis* causes gastric disease in humans.

Materials and Methods

Subjects and Specimen Collection. Gastric specimens were taken endoscopically from three *H. pylori*–negative patients: patient A (42-y-old female), patient B (52-y-old male), and patient C (45-y-old male). Ethical approvals were obtained from the ethics committee of Kyorin University School of Medicine and the medical research ethics committee of the National Institute of Infectious Diseases (NIID) for the use of human subjects. Written informed consent was obtained from all participants. *H. pylori* infection status in all patients was checked by urease breath test, rapid urease test, and pathological examination and confirmed to be negative by at least two tests. Gastric specimens from experimental pigs (11-wk-old), from a pig farm

in Japan, were provided by the Nippon Institute for Biological Science in Tokyo, Japan in December of 2019. All gastric specimens from human subjects and pigs were transported to NIID using NHPH transport media for culture, as described below.

***H. suis* Isolation and MIC Measurement.** As described previously (12, 33), NHPH medium consisting of *Brucella* broth (Difco Becton Dickinson), 20% fetal bovine serum (Gibco), Skirrow supplement (Oxoid), Vitox supplement (Oxoid), and 0.05% HCl (FUJIFILM Wako; final pH is ~5.0) was used for essentially all *H. suis* isolation procedures. NHPH transport media containing NHPH medium and 0.4% agar (Difco Becton Dickinson) was used for transportation of gastric biopsies. Transportation time from gastric biopsy collection to culture was about 6 h for patients A and B and 30 h for patient C. Homogenized gastric specimens were inoculated onto NHPH agar plates containing NHPH medium and 1.5% agar (Difco Becton Dickinson) and incubated under microaerobic conditions (5% O_2, 12% CO_2) in Forma Series II 3110 Water-Jacketed CO_2 Incubators (Thermo Fisher Scientific). Single colonies on NHPH agar plates were subcultured on agar plates and further enriched by modified biphasic culture. For the enrichment, cells were cultured for 3 to 5 d in a biphasic NHPH medium consisting of slant NHPH medium agar and broth in a culture flask with gentle shaking (50 rpm). Bacterial number was observed microscopically, and the broth part of the biphasic culture was exchanged by centrifugation until the bacterial number reached ~10^8 cells/mL All isolates were stocked in *Brucella* broth containing 30% glycerol at −80 °C for further experiments.

Susceptibility testing of *H. suis* isolates was performed by the broth microdilution method described in *SI Appendix, Supplemental Materials and Methods*. The MIC value was determined as the lowest concentration that inhibited bacterial growth. *H. suis* strain SNTW101c, recently isolated from mouse stomach, was also used in this study (14).

Genomic Sequencing and Comparative Genomic Analysis. Genomic DNA from *H. suis* isolates was extracted using Genomic-tips 20/G and buffers (Qiagen) and sequenced on the MiniSeq platform (Illumina) using High Output Reagent Kit (300 cycles) and on the MinION platform (Oxford Nanopore Technologies [ONT]) using the R9.4.1 flow cell. The library for Illumina sequencing (paired-end, insert size of 500 to 900 bp) was prepared using Nextera XT DNA Library Prep Kit (Illumina), and the library for ONT sequencing was prepared using the Rapid Sequencing Kit (SQK-RAD004). ONT reads were base called with Guppy version 3.4.4 (https://github.com/joshwcomeau/guppy). For complete genome analysis, obtained Illumina and ONT reads were hybrid-assembled de novo using Unicycler version 0.4.8 (https://github.com/rrwick/unicycler). For draft genome analysis, Illumina reads were assembled de novo using Shovill version 1.0.4 (https://github.com/tseemann/shovill). The resultant genome sequences were annotated using the DNA Data Bank of Japan (DDBJ) Fast Annotation and Submission Tool (https://dfast.ddbj.nig.ac.jp/) (*SI Appendix, Table S2*). All genome sequences have been deposited at GenBank/European Molecular Biology Laboratory/DDBJ under BioProject number PRJDB8704.

Pangenome analysis of *H. suis* using the 5 genomes in this study and 11 publicly available genomes from the Pathosystems Resource Integration Center database (https://www.patricbrc.org/) was performed using Roary version 3.13.0 (https://sanger-pathogens.github.io/Roary/) with default settings (minimum identity for Protein Basic Local Alignment Search Tool: 95%) and visualized using Phandango version 1.3.0 (https://jameshadfield.github.io/phandango). Phylogenetic analysis based on the core genome alignments of *H. suis* from the Roary pipeline was performed using Randomized Axelerated Maximum Likelihood version 1.0.0 (https://github.com/stamatak/standard-RAxML) with 1,000 bootstraps and visualized using FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Linear comparison of the chromosomal sequences of complete genomes of *H. suis*, including four genomes from this study, was performed using progressiveMauve version 2015_02_13.0 (http://darlinglab.org/mauve/mauve.html) with default settings. Genome

sequencing and single-nucleotide variant/indel detection analysis of *H. suis* isolates recovered from infected mice are described in *SI Appendix, Table S3*).

Animal Experiments. The 4-wk-old female C57BL/6NCrl mice (Charles River Japan) were infected intragastrically using a feeding needle with 1×10^8 colony-forming units of the indicated strain of *H. suis*. All mice were bred at the animal facility of the Kitasato Institute under an approved study protocol.

After 4 mo had passed following the infection, mice were killed, and the stomach and serum were collected from each mouse for evaluation. Mouse stomachs were cut along the greater curvature, rinsed with phosphate buffered saline (pH7.4) containing 0.01% (weight/volume) gelatin, and half of the stomach was subjected to histopathological examination. Longitudinal strips from the fundus, upper body, lower body, and antrum were fixed with 10% (wt/vol) neutral-buffered formalin (FUJIFILM Wako) and embedded in paraffin. Tissue sections ~4 μm thick were prepared and mounted on glass slides. The sections were stained with H&E and Alcian blue–PAS. The images were captured with a Nano Zoomer XR (Hamamatsu Photonics). Histopathological scoring was performed as shown in *SI Appendix, Table S4*.

For analyzing metaplastic changes, we stained the sections with TFF2 for SPEM and TFF3 for IM. The sections were deparaffinized with HistoClear (National Diagnostics) and quenched with an alcohol series, and then endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 3,3′-diaminobenzidine (DAB) staining. Antigen was retrieved by microwave heating for 20 min in an Immunosaver (Fujifilm Wako). For primary antibody conjugation, the section was blocked with serum and incubated overnight with anti-TFF2 antibody (1:100) [a kind gift from Sir Nicholas Wright (34)] or anti-TFF3 antibody (1:1,000) (UCLNT3, Thermo Fisher Scientific). The avidin-biotin complex (ABC) method (Vectastain ABC kit, Vector) was used to visualize staining. The Peroxidase–DAB system was used for TFF3 staining, and the Alkaline phosphatase–Vector Red system was used for TFF2 staining.

The other half of the stomach was homogenized in *Brucella* broth following treatment with 1% HCl for 45 min and then used for *H. suis* isolation (33) and DNA extraction. Relative *H. suis* count in mouse stomach was evaluated by the comparative ΔCt method using probe-based qPCR targeting the gene encoding the *H. pylori* VacA-like autotransporter protein (NHP194003_11930 in NHP19-4003, *SI Appendix, Supplemental Materials and Methods*).

Serum cytokines, chemokines, and *H. suis*–specific IgG were measured by the methods shown in *SI Appendix, Supplemental Materials and Methods*.

All statistical analyses were performed using GraphPad Prism 8.4.1 (La Jolla) as shown in *SI Appendix, Supplemental Materials and Methods*.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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