Current Research in Microbial Sciences 3 (2022) 100155

Contents lists available at ScienceDirect

Current Research in Microbial Sciences

journal homepage: www.sciencedirect.com/journal/current-research-in-microbial-sciences

Bacterial artificial chromosome-based reverse genetics system for cloning and manipulation of the full-length genome of infectious bronchitis virus

Yujin Inayoshi [a,*], Shiori Oguro [a], Erika Tanahashi [a], Zhifeng Lin [a], Yasushi Kawaguchi [b], Toshiaki Kodama [a], Chihiro Sasakawa [a,c]

[a] Nippon Institute for Biological Science, 9-2221-1 Shin-machi, Ome, Tokyo 198-0024, Japan
[b] Division of Molecular Virology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[c] Medical Mycology Research Center, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8673, Japan

ARTICLE INFO

Keywords:
Infectious bronchitis virus
Coronavirus
Reverse genetics system
Recombinant IBV
Spike protein
Vaccine

ABSTRACT

Avian infectious bronchitis virus (IBV) causes highly contagious respiratory reproductive and renal system diseases in chickens, and emergence of serotypic variants resulting from mutations in the viral S gene hampers vaccine management for IBV infection. In this study, to facilitate the molecular analysis of IBV pathogenesis and the development of a new-generation IBV vaccine, we established a reverse genetics system (RGS) for cloning the full-length cDNA of the IBV C-78E128 attenuated strain in a bacterial artificial chromosome (BAC). The BAC-cloned C-78E128 cDNA generated infectious viruses with biological properties of the parental C-78E128 strain with regard to an avirulent phenotype, tissue tropism and induction of virus neutralizing (VN) antibody *in vivo*. To assess the feasibility of genetic manipulation of the IBV genome using the BAC-based RGS, the S gene of the BAC-cloned C-78E128 cDNA was replaced with that of the IBV S95E4 virulent strain, which differs from the C-78E128 strain in serotype and tissue tropism, by bacteriophage lambda Red-mediated homologous recombination in *Escherichia coli* (*E. coli*). The resultant S gene recombinant virus was found to be avirulent and fully competent to induce a serotype-specific VN antibody against the S95 strain; however, the S gene recombinant virus did not fully recapitulate the tissue tropism of the S95E4 strain. These data imply that serotype-specific VN immunogenicity, but not tissue-tropism and pathogenicity, of IBV is determined by the viral S gene. The IBV BAC-based RGS that enables cloning and manipulation of the IBV virus genome entirely in *E. coli* provides a useful platform for the molecular analyses of IBV pathogenesis and the development of rationally designed IBV recombinant vaccines.

Abbreviations

BAC	bacterial artificial chromosome
EID_{50}	50% egg infectious dose
FP	fusion peptide
IBV	infectious bronchitis virus
KBD	kidney binding domain
nsp	nonstructural protein
ORF	open reading frame
PBS	phosphate buffered saline
RGS	reverse genetics system
RT	reverse transcriptase
PCR	polymerase chain reaction
SPF	specific pathogen free
TBD	trachea binding domain
VN	virus neutralization/virus neutralizing
VR	variable region

1. Introduction

Avian infectious bronchitis virus (IBV) is the etiological agent of highly contagious respiratory diseases such as bronchitis and tracheal ciliostasis in domestic fowl, and causes serious economic loss in the poultry industry worldwide. IBV replicates primarily in epithelial cells of the upper respiratory tract, while IBV infection frequently involves epithelial surfaces of the kidney, gut, and oviduct and causes nephritis,

* Corresponding author.
E-mail address: inayoshi@nibs.or.jp (Y. Inayoshi).

https://doi.org/10.1016/j.crmicr.2022.100155

Available online 13 July 2022

intestinal disorders, and decreased egg production (Cavanagh, 2007; Jackwood, 2012).

IBV, a member of the genus *Gammacoronavirus* in the family *Coronaviridae,* order *Nidovirales*, is an enveloped virus with an unsegmented, single-stranded positive sense 27.6 kb RNA genome. The 5′ two-thirds of the IBV genome comprises open reading frame (ORF) 1a/1ab which encodes 15 nonstructural proteins (nsp2 to 16) involved in virus RNA transcription, replication and polyprotein processing. The remaining one-third of the 3′ genome encodes structural proteins, *i.e.*, spike (S), small membrane (E), integral membrane (M), nucleocapsid (N), and four accessory proteins (ORFs 3a, 3b, 5a, 5b). Untranslated regions (UTRs) approximately 500 nucleotides in length at the 5′- and 3′- termini of the IBV RNA genome called 5′UTR and 3′UTR, respectively, are predicted to form stem-loop structures that are important for transcription and replication of viral RNA. The IBV genome is organized as follows: 5′UTR-1a/1ab-S-3a-3b-E-M-5a-5b-N-3′UTR (Cavanagh, 2007; Dalton et al., 2001).

The genome of IBV, like that of other RNA viruses, exhibits substantial genetic diversity due primarily to an error-prone RNA-dependent RNA polymerase. The evolution of genetic mutants and selective pressure *in vivo* leads to the emergence of phenotypic variants differing in cell/tissue tropism, pathogenicity, and immunogenicity. In the IBV genome, the S gene, encoding S glycoprotein that initiates viral infection of host cells, is the most highly divergent, presumably as a consequence of immune selection pressure *in vivo* (Jackwood et al., 2012; Wickramasinghe et al., 2014). S glycoprotein, a highly glycosylated class I viral fusion protein, is post-translationally cleaved to an amino-terminal S1 subunit and a carboxyl-terminal S2 subunit, which function in receptor binding and virus–cell membrane fusion, respectively (Masters, 2006; Wickramasinghe et al., 2014). Among the various IBV strains, the S1 subunit is the most divergent in amino acid sequence, while the S2 subunit is relatively well-conserved (Wickramasinghe et al., 2014). The S1 subunit represents a receptor binding region and major antigenic epitopes for virus neutralizing (VN) antibody, so mutations in the S1 subunit are one of the major factors that drive the evolution of variant viruses with distinct cell/tissue tropism and serotype specificity (Cavanagh et al., 1988; Kant et al., 1992; Promkuntod et al., 2014; Shan et al., 2018). Based on S1 gene sequences, phylogenetic analysis has identified several genotypes (Jackwood, 2012).

Although vaccination is the most common preventive measure for IBV infection, the emergence of serotypic variants that are not cross-reactive to commercial vaccines (*i.e.*, vaccine breakthrough) makes it difficult to control the spread of IBV (de Wit et al., 2011). It is ideal to develop IBV vaccines that are effective against currently prevalent IBV strains; however, the development of live attenuated IBV vaccine is not only time-consuming but also unpredictable with regard to attenuation and vaccine efficacy. To overcome the limitations associated with current IBV vaccination methods, an innovative approach is highly desirable for IBV vaccine development.

A reverse genetics system (RGS) that rescues infectious virus entirely from cloned full-length viral genome and allows genetic modification of the cloned virus genome, provides a powerful and straightforward tool for basic research and genetic vaccine development (Racaniello and Baltimore, 1981; Rice et al., 1989). An RGS for coronavirus, however, is challenging due to instability of its large viral genome in *Escherichia coli* (*E. coli*), with previous studies using *in vitro* ligation of viral cDNA fragments and recombination in a vaccinia vector to construct a full-length viral genome (Casais et al., 2001; Thiel et al., 2001; Yount et al., 2000). Bacterial artificial chromosomes (BACs) are well-established for stably maintaining large cloned DNAs as a single copy in *E. coli*, and a BAC-based RGS has been used for the genomic manipulation of herpesvirus, which has a genome larger than 120 kb (Messerle et al., 1997; Shizuya et al., 1992), and various coronavirus species (Almazán et al., 2006; Almazán et al., 2013, 2000; Balint et al., 2012; Lv et al., 2020).

In this study, we report a BAC-based RGS for cloning the full-length cDNA of IBV attenuated C-78E128 strain to rescue infectious cloned virus. For genetic modification of the BAC-cloned viral genome, we applied the bacteriophage lambda Red-mediated two-step homologous recombination system which precisely and seamlessly alters target genes at a desired position in full-length viral cDNA in *E. coli* (Jarosinski et al., 2007; Tischer et al., 2006). The feasibility of this BAC-based RGS for the development of an IBV genetic vaccine was proven by the successful generation of an S gene recombinant IBV simply by swapping the S gene of the BAC-cloned C-78E128 with that of a virulent IBV S95E4 field strain differing in serotype and tissue tropism.

2. Materials and methods

2.1. Viruses

Virulent C-78E6 and attenuated C-78E128 strains had been developed by 6- and 128- times serial passage of a primary isolate of the C-78 strain, respectively, in embryonated specific pathogen-free (SPF) chicken eggs (Nisseiken Co., Ltd, Ome, Japan) (Lin et al., 1991). Similarly, virulent S95E4 and attenuated S95E52 strains were obtained by 4- and 52-times serial passage of a primary S95 isolate in eggs, respectively. The full-length viral genome of C-78E128 (GenBank/NCBI/NLM accession number LC663496) was used for BAC cloning. The 5′- and 3′-terminal sequences of the C-78E128 genome were determined using a 5′/3′RACE kit (Roche, Basel, Switzerland). The S gene of S95E4 (GenBank/NCBI/NLM accession number LC663499) was used for S gene recombination. The S genes of the C-78E6 and S95E52 strains (GenBank/NCBI/NLM accession numbers LC663497 and LC663498, respectively) were used for a comparative analysis.

2.2. Construction of BAC transcription vector

DNA fragments containing human cytomegalovirus (CMV) immediate early promoter and simian virus 40 (SV40) early mRNA polyadenylation (polyA) signal were PCR-amplified from pEGFP-C1 (Takara Bio Inc., Kusatsu, Japan) (GenBank/NCBI/NLM accession U55763). The PCR amplicons were cloned into the SphI site of pBeloBAC11 (New England Biolabs Inc. Ipswich, MA) (GenBank/NCBI/NLM accession number U51113) using an In-Fusion cloning kit (Takara Bio Inc.) to construct a pBAC transcription vector. The primer sets used for pBAC construction are listed in supplemental materials (sTable 1).

2.3. In-Fusion cloning of full-length IBV cDNA in pBAC transcription vector

Total RNA was prepared from IBV C-78E128 virus stock using a pure RNA isolation kit (Roche), and viral cDNA was synthesized using random hexa-deoxyribonucleotide primers and a PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio Inc.). The full-length cDNA of the C-78E128 genome was amplified as eight contiguous cDNA fragments, designated F1 ~ F8, that contained primer-designed overlapping sequences at the 5′ end and overlapping sequences and a SacII restriction site at the 3′ end (Fig. 1A) by using the primer sets listed in supplemental materials (sTable 2).

For cloning full-length cDNA into the pBAC transcription vector, the 5′-terminal F1 cDNA fragment was initially cloned into the SphI site of pBAC; then, F2 ~ F8 contiguous cDNA fragments were directionally assembled into the SacII site of pBAC stepwise by using an In-Fusion cloning kit (Takara Bio Inc.) (Fig. 1B). Sequence analysis of the resultant BAC clone containing full-length C-78E128 cDNA, named pBAC/C-78FL, revealed eight and one mismatch in the ORF1ab and N genes, respectively, compared to the parental C-78E128 strain.

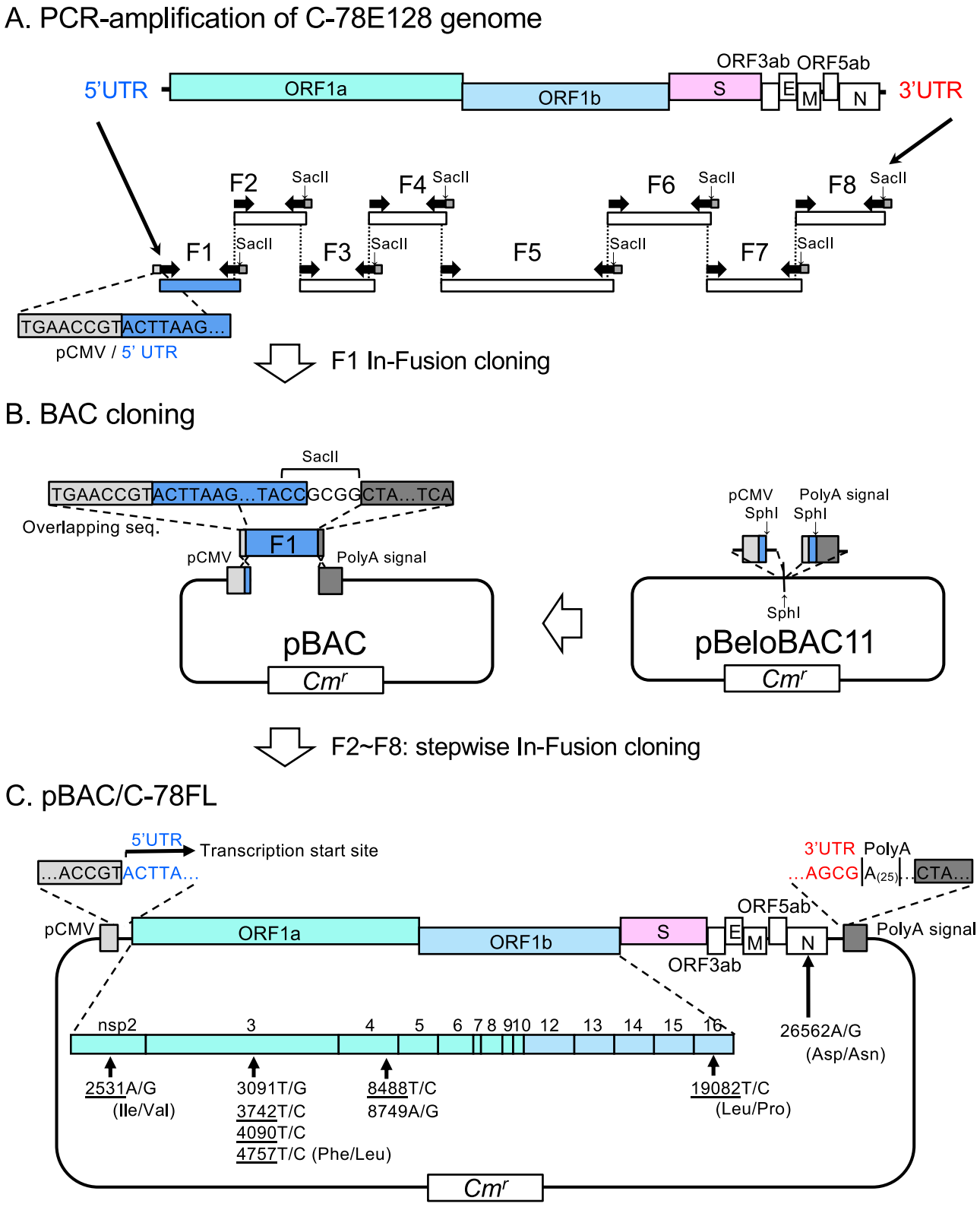


Fig. 1. Cloning strategy for full-length C-78E128 cDNA in a BAC. (A) PCR amplification of eight contiguous cDNA fragments from C-78E128 cDNA (F1 ~ F8) containing overlapping sequences and a SacII site (thin arrows). The bold arrows indicate primer sets used for amplification (supplementary sTable 2). Sequences of 5′primer containing pCMV and 5′UTR sequences used for cloning F1 fragment are shown. UTR, untranslated region; ORF, open reading frame; S, spike protein gene; M, membrane protein gene; E, envelope protein gene; N, nucleocapsid protein gene. (B) Construction of pBAC transcription vector and stepwise In-Fusion cloning of the full-length IBV genome. pCMV, PolyA signal, and *Cm*^{r} indicate CMV promoter, SV40 early mRNA polyadenylation signal, and chloramphenicol resistance gene, respectively. (C) Schematic of pBAC/C-78FL and mismatch sites corresponding to the C-78E128 genome sequence. Transcription start site at the 5′UTR and a junction of the 3′UTR with PolyA signal in pBAC/C-78FL are shown. Light blue boxes and bold arrows show the nonstructural protein genes (nsps) of ORF1ab and genes containing mismatch sites, respectively. Repaired nucleotide positions are underlined, and amino acid changes caused by nonsynonymous mutations are indicated in parentheses.

2.4. Genetic manipulation of BAC-cloned full-length viral genome in E. coli

We employed bacteriophage lambda Red-mediated two-step homologous recombination to manipulate the BAC-cloned full-length IBV genome in *E. coli* (Kato et al., 2008; Tischer et al., 2006). The BAC-cloned DNA was transfected into *the E. coli* GS1783 strain (Tischer et al., 2010), which carries chromosomally encoded genes for Red-mediated recombination under a temperature-inducible promoter, and the I-SceI gene under an arabinose-inducible promoter. To construct a gene transfer vector for Red-mediated recombination, named pBR-kan, the I-SceI recognition site and the kanamycin resistance gene (Km^r) were amplified from pEPkan-S (Tischer et al., 2006) by using primer set Isce-kan-pF/Isce-kan-pR (sTable 1) and cloned into the EcoRI/BamHI sites of pBR322 (Nippon gene Co., Ltd, Tokyo, Japan) (GenBank/NCBI/NLM accession J01749.1). IBV sequences of interest were cloned into the pBR-kan transfer vector, and a DNA fragment containing IBV sequences, the I-SceI recognition site, and Km^r was amplified and transfected into the *E. coli* GS1783 strain harboring the BAC-cloned DNA to initiate the 1st Red-mediated recombination reaction. Km^r was then excised via arabinose-induced cleavage at the I-SceI site and the 2nd Red-mediated recombination in *E. coli.*

2.5. Repair of sequence mismatches in the ORF1ab gene of pBAC/C-78FL

To repair the sequence mismatches found in the ORF1ab gene in pBAC/C-78FL, ORF1ab fragments of interest were amplified from the parental C-78E128 cDNA and cloned into the EcoRI and BamHI sites of the pBR-kan transfer vector using the In-Fusion cloning kit. For Red-mediated recombination, PCR product containing the ORF1ab fragments, I-SceI site, and Km^r was amplified and transfected into the *E. coli* GS1783 strain harboring pBAC/C-78FL. Red-mediated recombination was repeatedly performed to repair the eight sequence mismatches in ORF1ab, eventually yielding a clone, named pBAC/C-78Rep, with six out of the eight mismatches repaired correctly. The procedure and primer sets used for the mismatch repair are shown in supplementary materials (Fig. S1 and sTable 3).

2.6. Construction of the S gene recombinant

The S gene of pBAC/C-78Rep was replaced with that from the virulent IBV S95E4 strain using homologous Red-mediated recombination. To facilitate S gene recombination, we initially deleted the entire S gene from pBAC/C-78Rep: DNA fragments from 3′ ORF1b and 5′ ORF3a lacking the S gene in C-78E128 were synthesized and cloned into the pBR-kan transfer vector. DNA containing the I-SceI site, 3′ ORF1b/5′ ORF3a, and Km^r was then amplified and transfected into the *E. coli* GS1783 strain harboring pBAC/C-78Rep to create S gene-deleted pBAC/C-78RepΔS.

To swap the S gene of the S95E4 virulent strain with that of C-78E128, the S1 and S2 genes were separately amplified from S95E4 cDNA and cloned into the pBR-kan transfer vector. DNA fragments containing the S1 and S2 genes of S95E4, the I-SceI site and Km^r were amplified and transfected into GS1783 harboring pBAC/C-78RepΔS to construct S gene-recombinant pBAC/C-78Rep/S95S. The procedures and primer sets used for S gene deletion and recombination in *E. coli* using two-step Red-mediated recombination are shown in supplementary materials (Fig. S2 and sTables 4 and 5).

2.7. Virus rescue and amplification from BAC-cloned IBV cDNA

The constructed pBAC/C-78FL, pBAC/C-78Rep and pBAC/C-78Rep/S95S were transfected into 293T cells (kindly provided by K. Nishigaki, Yamaguchi University) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, Mass.) to rescue the BAC-cloned viruses, named rC-78FL, rC-78Rep, and rC-78Rep/S95S, respectively. To amplify the rescued virus, cell suspensions prepared from the transfected 293T cells were inoculated in 9- to 10-day-old embryonated SPF chicken eggs. After two to three days of incubation, allantoic fluid was harvested, filtered through a 0.45 μm membrane filter (Millipore, Merck Life Science, Darmstadt, Germany), and stocked as first egg passage (E1) virus. The second passage (E2) was performed by inoculating 0.1 ml of E1 virus in embryonated SPF chicken eggs, and E2 allantoic fluid was harvested and used to characterize the biological properties of BAC-cloned viruses. Sequences of the cloned viruses were confirmed by sequence analysis. No mutation was detected compared to the sequences of the viral cDNA cloned into pBAC.

2.8. In vivo experimental infection with IBV

Experimental infection of SPF chickens with IBV was approved by the Institutional Animal Experimental Committee (permission No. 14–068B, 15–043B, and 16–059B) and performed in separate HEPA filter-equipped isolators according to the Institutional animal experimentation regulations. Four-day-old SPF chickens were inoculated once via eye-drop with C-78E128 attenuated strain ($n = 10{\sim}12$, $10^{4.5}$ EID_{50}/dose), C-78E6 virulent strain ($n = 10$, $10^{4.5}$ EID_{50}/dose), S95E52 attenuated strain ($n = 9$, $10^{4.5}$ EID_{50}/dose), and S95E4 virulent strain ($n = 20$, $10^{4.5}$ EID_{50}/dose), or the BAC-derived cloned IBV rC-78FL ($n = 12$, $10^{4.1}$ EID_{50}/dose), rC-78Rep ($n = 12$, $10^{4.5}$ EID_{50}/dose), or rC-78Rep/S95S ($n = 9$, $10^{4.5}$ EID_{50}/dose). The IBV-infected chickens were reared for up to 28 days to monitor clinical signs, and blood samples were taken from the wing on the final day of housing to measure serum neutralization antibody. Sera from non-immunized chickens were used as control. To analyze virus distribution *in vivo,* four-day-old chickens ($n = 2$) were separately infected with the viruses in the same manner as above and tissue samples were collected at 7 days post-inoculation (p.i.).

2.9. RT-PCR analysis to detect the IBV S gene

Total RNA was extracted from the cells and culture supernatant of 293T cells transfected with the BAC-cloned IBV cDNA, the allantoic fluid of virus-infected embryonated chicken eggs, and supernatants of 10% tissue homogenates of IBV-infected chickens by using the QuickGene RNA tissue kit S II (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) according to the manufacturer's instructions. To eliminate contamination of the BAC-cloned IBV cDNA used for transfection, RNA prepared from the transfected 293T cells was treated with RQ1 RNase-free DNase (Promega, Madison, WA) and then RNAs were concentrated by isopropanol precipitation. RNA samples were reverse transcribed by SuperScript II Reverse Transcriptase (Thermo Fisher Scientific), and a 400 bp fragment from the IBV S2 subunit was amplified using PrimeSTAR Max Polymerase (Takara Bio Inc.) as previously reported (Kato et al., 2019; Lin et al., 1991).

2.10. Neutralization assays

Virus neutralization (VN) assays were performed using the immunoperoxidase monolayer assay, as described previously (Koch et al., 1986) with a minor modification. Briefly, four-fold serial dilutions of test serum were mixed with 100 $TCID_{50}$ of IBV and inoculated into SPF chicken kidney (CK) cells in a 96-well microplate. After 2 days of virus infection, IBV-infected CK cells were visualized by immunostaining using a monoclonal antibody against IBV N protein and HRP-labeled goat anti-mouse IgG (MP Biomedicals, LLC, Irvine, USA) with 3,3'-diaminobenzidine tetrahydrochloride as a peroxidase substrate (Chhabra et al., 2016). A 50% or more reduction in virus infection was considered as significant VN, and 50% endpoint neutralizing serum titers were calculated by the Spearman-Kärber method (Kärber, 1931; Spearman, 1909). For VN assays using CK cells, C-78, and S95 strains adapted to replicate in CK cells were used.

3. Results

3.1. Cloning of the full-length IBV genome into a BAC

To establish an RGS for IBV, we cloned the entire genome of the attenuated C-78E128 strain into a BAC. To facilitate cloning and assembly of the full-length virus genome in the BAC, the viral cDNA was amplified as eight contiguous cDNA fragments, designated F1 ~ F8, containing primer-designed overlapping sequences at the 5′ and overlapping sequences and a SacII restriction site at the 3′ end (Fig. 1A). The 5′-terminal F1 cDNA fragment containing the viral 5′UTR was initially cloned into the SphI site of a pBAC transcription vector that was constructed by cloning the CMV promoter and SV40 poly A signal into the SphI site of a pBeloBAC11 cloning vector. Then, the F2 ~ F8 contiguous cDNA fragments were directionally assembled into the SacII site of the pBAC vector via stepwise In-Fusion cloning (Fig. 1B).

Compared to the consensus sequence of C-78E128, the resultant BAC clone, named pBAC/C-78FL, had eight nucleotide mismatches within the ORF 1ab (transcriptase/replicase) gene and one mismatch in the N (nucleocapsid) gene (Fig. 1C). Among these mismatches, three at nucleotides (nts) 2531, 4757, and 19,082 in the ORF1ab gene and one at nt 26,562 in the N gene were non-synonymous, while the remaining mismatches were synonymous. Sequencing chromatograms of C-78E128 cDNA showed a minor peak corresponding to the mismatches found in pBAC/C-78FL (data not shown). This suggests genetic heterogeneity of the C-78E128 attenuated strain, from which a minor quasispecies existing in C-78E128 might be PCR-amplified and cloned into pBAC/C-78FL.

3.2. Rescue of infectious C-78FL clone

Although pBAC/C-78FL did not fully represent the consensus sequence of C-78E128, virus recovery from the BAC-cloned IBV cDNA was attempted. To rescue viruses from cDNA, pBAC/C-78FL DNA was transfected into 293T cells (Fig. 2A). pBAC plasmid was also transfected as a negative control. Two days after transfection, transient virus production in the transfected cells and culture supernatant was examined by RT-PCR. RNA purified from the samples was treated with DNase to digest the pBAC/C-78FL DNA used for the transfection. Results from RT-PCR of the IBV S2 gene showed a relatively weak but specific

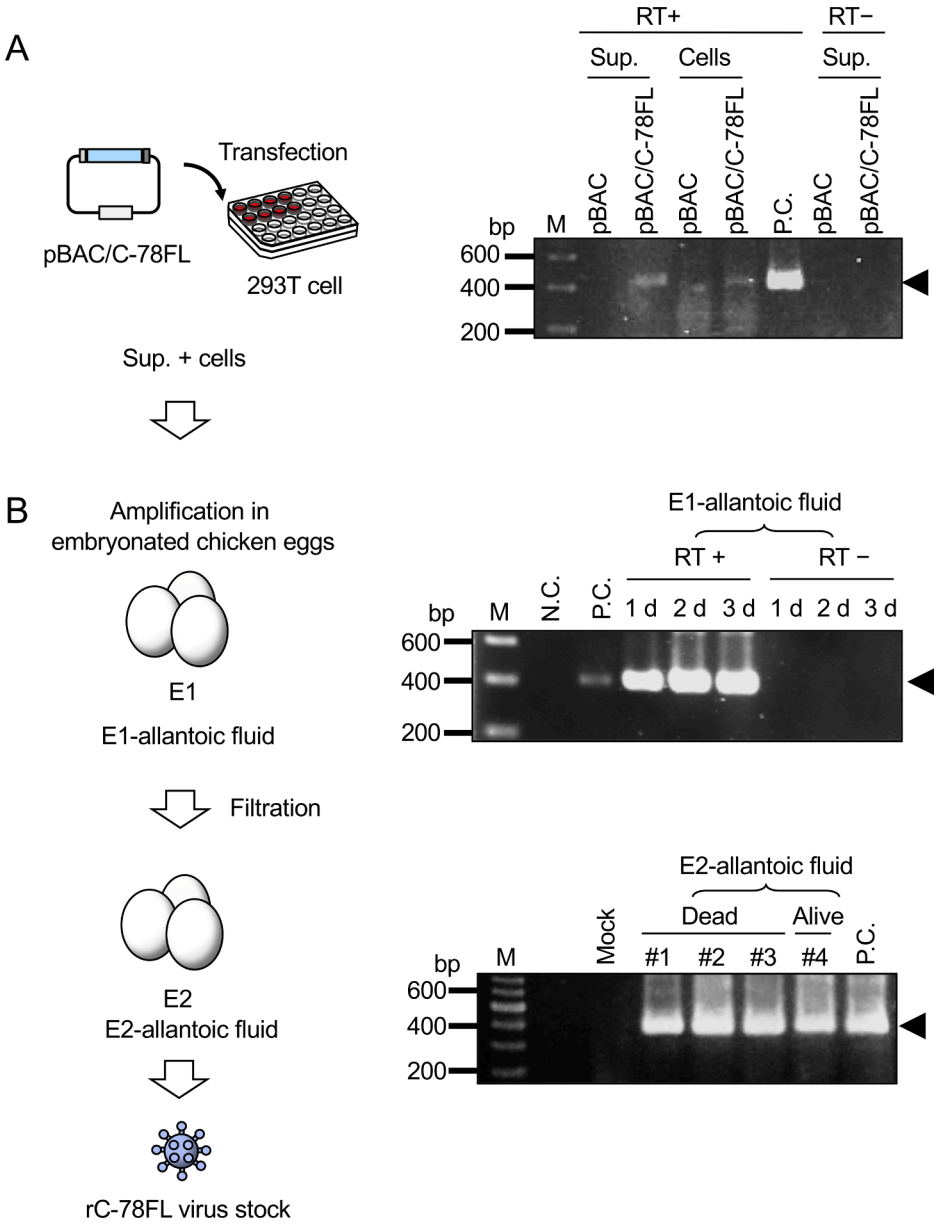


Fig. 2. Rescue and amplification of BAC-cloned rC-78FL. (A) Transfection of pBAC/C-78FL into 293T cells, and RT-PCR analysis of culture supernatant (Sup.) and transfected cells (Cells) separately harvested. RT− indicates omission of the RT reaction from the RT-PCR. RT-PCR-amplified S2 gene yielding a 400 bp band is indicated by the arrowhead. The amplification products were electrophoresed on 2% agarose gel. M, 200 bp DNA ladder; pBAC, vector control; P.C., diluted C-78 virus stock. (B) Amplification of rC-78FL using embryonated chicken eggs. RT-PCR analysis of allantoic fluid from egg passage 1 (E1). E1-allantoic fluid harvested 1, 2, and 3 days after inoculation (1, 2 and 3 d) was subjected to RT-PCR. N.C. indicates allantoic fluid from eggs inoculated with control vector-transfected cells. (C) Preparation of rC-78FL virus stock. E1-allantoic fluid was inoculated into E2 embryonated eggs and RT-PCR was performed using E2-allantoic fluid; Dead indicates allantoic fluid of dead chicken embryo until 2 days p.i. and Alive indicates inoculated embryo at 3 days p.i. Mock indicates allantoic fluid inoculated with N.C. from Fig. 2B. M represents 100 bp DNA ladder.

amplification of a 400 bp fragment in both the cells and supernatant from the pBAC/C-78FL transfection, but not those from the pBAC control transfection (Fig. 2A). When the reverse transcriptase (RT) reaction was omitted from the RT-PCR, no amplification was found in the supernatant of the pBAC/C-78FL transfectant, indicating no contaminating transfected DNA was present in the RT-PCR analyses.

To examine if the pBAC/C-78FL-transfected 293T cells produce infectious viruses, cells and supernatant harvested 2 days after transfection were mixed and inoculated into the allantoic cavity of embryonated chicken eggs (E1; Fig. 2B). RT-PCR analysis of allantoic fluid collected at 1, 2, and 3 days p.i. showed robust amplification of the IBV S2 gene, but no amplification was detected when the RT reaction was omitted.

To prepare cell-free virus stock, allantoic fluid from E1 obtained at 3 days p.i. was filtered through a 0.45 μm membrane filter and inoculated into four embryonated chicken eggs (E2. Fig. 2C). Three embryos (#1 ~ 3) died at 2 days p.i. and the #4 embryo survived up to 3 days p.i., with RT-PCR analysis showing strong amplification of the S2 gene fragment in all E2 allantoic fluid samples. Inoculation of the E2 allantoic fluid into embryonated chicken eggs resulted in characteristic IBV pathogenic effects in the embryo, such as stunting and curling (data not shown). These results indicate that the pBAC/C-78FL clone is fully competent to generate infectious progeny viruses that replicate and induce pathogenic effects in chicken embryo. The E2 allantoic fluid containing pBAC/C-78FL-derived cloned virus, designated rC-78FL, was used for the following viral characterization experiments *in vivo*.

3.3. Characterization of the IBV rC-78FL clone

Twelve four-day-old SPF chickens were experimentally infected with rC-78FL to analyze viral pathogenicity and immunogenicity *in vivo*. As a comparison, SPF chickens were also infected with either the C-78E6 virulent strain ($n = 10$) or the parental C-78E128 attenuated strain ($n = 10$) derived from serial passages of the virulent C-78 primary isolate in embryonated chicken eggs. Mock-infected chicken sera did not show VN activity against the C-78 strain.

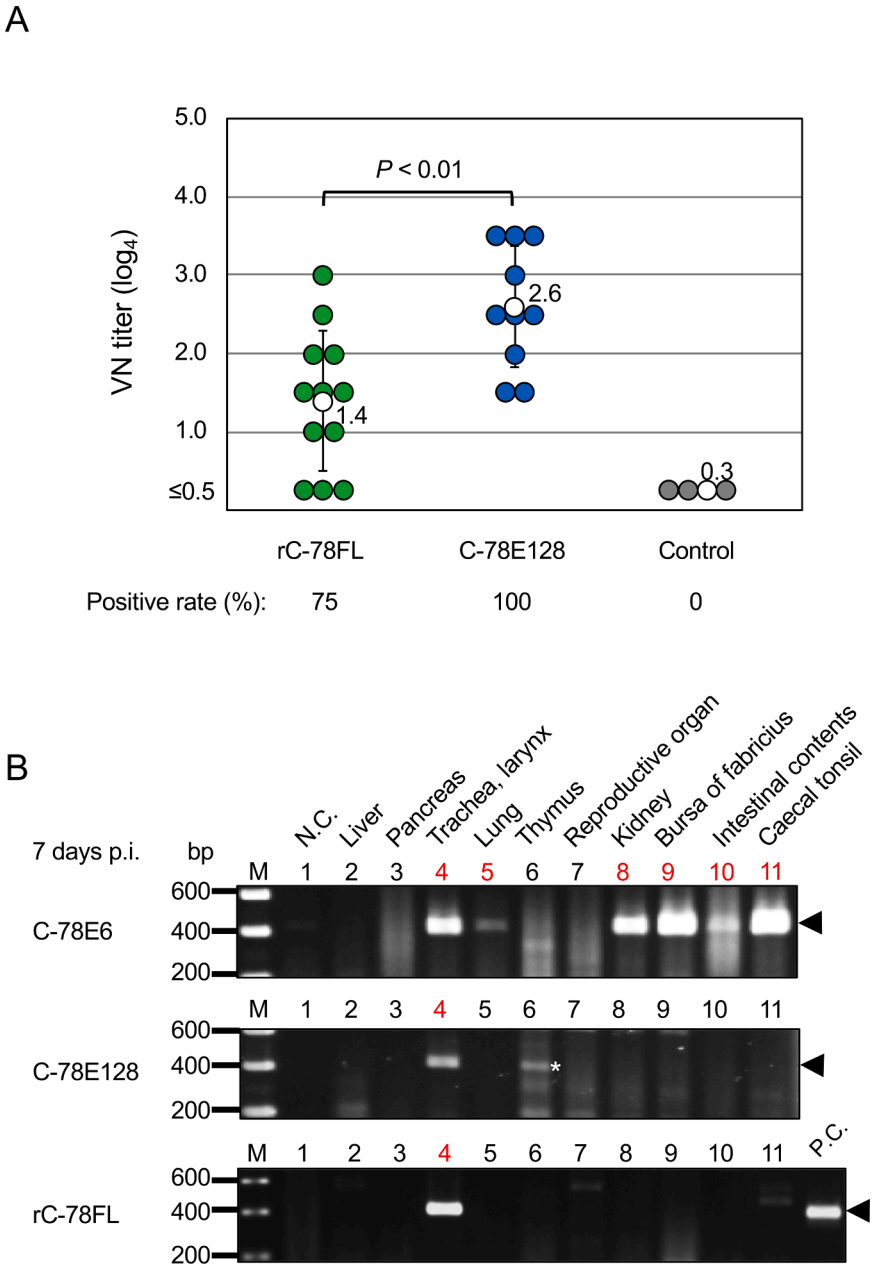


Fig. 3. Immunogenicity and tissue-tropism of rC-78FL. (A) Virus neutralization (VN) titers of chickens inoculated with rC-78FL ($n = 12$) or C-78E128 attenuated strain ($n = 10$) against the C-78 strain. The individual antibody titers and geometric mean are shown by colored circles and open circles, respectively, as logarithmic values (logarithm base 4). Sera from three mock-infected chickens were used as control. Neutralization titers under the detection limit were calculated as $4^{0.25}$. Error bars represent standard deviation. Student's-*t*-test was used to calculated statistical significance. Positive rates indicate the percentage of C-78-antibody positive chickens in an individual group. (B) RT-PCR analysis of rC-78FL tissue-tropism. Amplification products were electrophoresed on a 2% agarose gel. The ~400 bp visual amplicons (arrowheads) were gel-purified and subjected to sequence analysis. The red number lanes indicate IBV S2 specific amplification and the asterisk in the center image indicates non-specific amplification. N.C. indicates PBS negative control.

During the 28-day observation period, all of the chickens infected with the C-78E6 virulent strain survived but displayed respiratory clinical signs, such as snicking, discharge from the nostrils and wheezing. In contrast, all of the chickens infected with rC-78FL, as well as those infected with the C-78E128 attenuated strain, remained healthy and did not show any clinical signs, indicating an attenuated phenotype for the rC-78FL virus (data not shown).

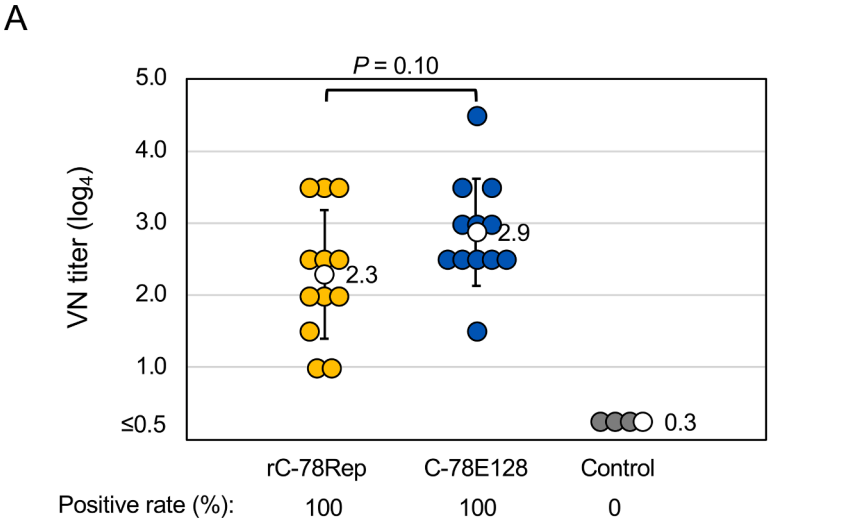
Serum samples taken from chickens infected with C-78E128 and rC-78FL at 28 days p.i. were analyzed for VN titer against a homologous C-78 strain. As shown in Fig. 3A, all chickens infected with the C-78E128 attenuated strain raised VN antibody, whereas 3 out of 12 chickens infected with the rC-78FL failed to do so. Average VN titer induced by rC-78FL ($4^{1.4}$) was significantly lower than that induced by C-78E128 ($4^{2.6}$) (P-value < 0.01). In repeated experiments, rC-78FL frequently failed to induce VN antibody in some chickens, and the average VN titer did not exceed 4^2.

Two four-day-old SPF chickens were additionally infected with either C-78E6, C-78E128 or rC-78FL, and virus distribution at 7 days p.i. in major organs and intestinal contents was analyzed by RT-PCR. A representative result from the RT-PCR analysis shown in Fig. 3B indicates that the C-78E6 virulent strain distributed to the upper respiratory tract (larynx and trachea), lung, kidney, bursa of Fabricius, caecal tonsil, and intestinal contents, whereas distribution of C-78E128 and rC-78FL were restricted to the upper respiratory tract. These data indicate that the rC-78FL cloned virus recapitulates the attenuated phenotype and tissue-tropism of the parental C-78E128 attenuated strain.

3.4. Repair of sequence mismatches in the ORF1ab gene of pBAC/C-78FL

We speculated that the sequence mismatches present in the rC-78FL virus genome might be responsible for the observed weak immunogenicity. Among a total of nine mismatches in the virus genome, eight were located in the ORF1ab gene. To address if the mismatches in the ORF1ab gene affected immunogenicity of rC-78FL, we attempted to repair the mismatches in the ORF1ab gene in the full-length pBAC/C-78FL genome in *E. coli* using two-step Red-mediated homologous recombination (Fig. S1). The resultant BAC clone, designated pBAC/C-78Rep, showed successful repair of the mismatches at nts 2531, 3742, 4090, 4757, 8488, and 19,082, but not those at nts 3091 and 8749 (Fig. 1C). However, since the mismatches at nts 3091 and 8749 were synonymous, we proceeded with virus rescue and characterization.

Virus stock derived from pBAC/C-78Rep, designated as rC-78Rep, was prepared by 293T cell transfection followed by passaging twice in embryonated chicken eggs. To examine the *in vivo* biological properties of rC-78Rep, 12 four-day-old SPF chickens were infected with the virus. During the 28-day observation period, the rC-78Rep-infected chickens showed no clinical signs characteristic of IBV infection. Assessment of the immunogenicity of rC-78Rep showed that all virus-infected chickens developed VN antibody against the C-78 strain, with an average VN titer of $4^{2.3}$, which is comparable to that induced by the C-78E128 attenuated strain ($4^{2.9}$) (Fig. 4A). This result indicates that the mismatch repair of the ORF1ab gene significantly improved virus immunogenicity. RT-PCR analysis of tissue distribution showed that, similar to C78E128 and rC-78FL (Fig. 3B), rC-78Rep was detected only in the larynx and trachea, but not other organs or intestinal contents (Fig. 4B). Thus, the biological properties of rC-78Rep in the context of virus pathogenicity, tissue-



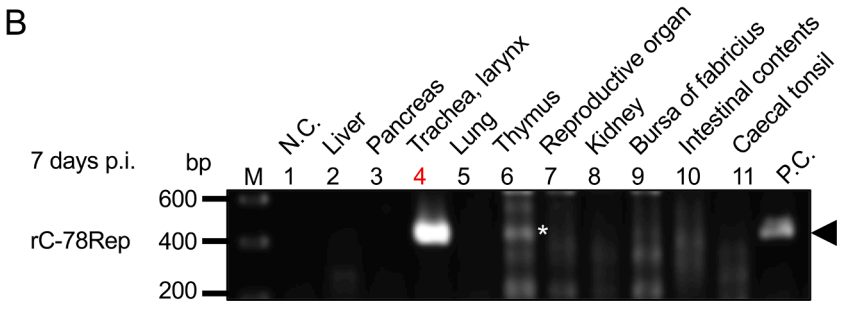


Fig. 4. Immunogenicity and tissue-tropism of rC-78Rep. (A) VN titers of chickens inoculated with rC-78Rep or C-78E128 (n = 12). The individual antibody titers and geometric mean are shown by colored circles and open circles, respectively, as logarithmic values (logarithm base 4). Sera from three non-immunized chickens were used as control. Error-bars indicate standard deviation. Student's t-test was used to calculated statistical significance. Positive rates (%) indicate the percentage of C-78-neutralizing antibody positive chickens in an individual group. (B) RT-PCR analysis of rC-78Rep tissue-tropism. Representative data of virus distribution in tissues of chickens infected with rC-78Rep at 7 days p.i. are shown. The ~400 bp visual amplicons (arrowheads) were gel-purified and subjected to sequencing analysis. The red numbered lane indicates S2 specific amplification, and the asterisk indicates non-specific amplification. N.C. indicates PBS negative control.

specificity, and immunogenicity were virtually indistinguishable from those of the parental C-78E128 attenuated strain. While rC-78Rep retained nucleotide mismatches at nts 3091 and 8749 in the ORF1ab gene and at nt 26,562 in the N gene, no further repair was attempted, and they were instead used as genetic markers.

3.5. Construction of S-gene recombinant virus

The BAC-cloned pBAC/C-78Rep that generates avirulent and immunogenic cloned viruses could serve as a platform to genetically construct a live vaccine against new IBV variants. To explore the feasibility of genetic manipulation of the BAC-cloned IBV genome for vaccine development, we sought to construct a recombinant virus by replacing the S gene of pBAC/C-78Rep with that of a virulent field isolate of the S95E4 strain. Genetically, C-78 and S95 belong to the same JP-I genotype but VN antibody against the C-78 strain shows weak cross-reactivity with the S95 strain.

The S gene of pBAC/C-78Rep was replaced with that of the virulent S95E4 strain via stepwise cloning using Red-mediated recombination in *E. coli*. To facilitate S gene recombination, the S gene was deleted from pBAC/C-78Rep by transfection of a DNA fragment containing 3′ ORF1b/5′ ORF3a, I-SceI site and Km^r into *E. coli* GS1783 strain harboring pBAC/C-78Rep for Red recombination. The resultant S gene-deleted pBAC/C-78RepΔS served as a recipient vector for S gene recombination (Figs. 5A and S2A). To construct the S gene recombinant, the S1 and S2 genes were separately amplified from S95E4 cDNA, and a DNA fragment containing the S1 and S2 gene of S95E4, the I-SceI site and Km^r was transfected into *E. coli* harboring pBAC/C-78RepΔS for Red-mediated recombination (Figs. 5B and S2B). Sequence analysis of the resultant BAC clone, designated pBAC/C-78Rep/S95S, showed that the S gene of the virulent S95 strain correctly recombined in the BAC/C-78Rep genetic background.

3.6. Characterization of the S gene recombinant virus

Virus stock from the pBAC/C-78Rep/S95S, designed rC-78Rep/S95S, was generated by transfection of 293T cells and propagation following two passages in embryonated chicken eggs. The biological properties of rC-78Rep/S95S were compared in parallel with those of the parental S95E4 virulent strain and an S95E52 attenuated strain that had been developed from 52-times serial passage of the virulent S95 primary isolate in chicken embryo.

Four-day-old chickens were infected with either the rC-78Rep/S95S ($n = 9$), virulent S95E4 ($n = 20$), or attenuated S95E52 strain ($n = 9$). During the 28-day observation period, all the chickens infected with the virulent S95E4 strain displayed clinical signs characteristic of IBV infection (snicking, discharge from the nostrils, wheezing, diarrhea, and depression) as early as 4 days p.i., and nine of the S95E4-infected chickens died as early as 8 days p.i. (fatality rate: 45%). In contrast, neither chickens infected with the attenuated S95E52 strain nor rC-78Rep/S95S displayed clinical signs characteristic of IBV infection (data not shown).

Serum samples taken from the chickens infected with the rC-78Rep/S95S recombinant and attenuated S95E52 strain at 28 days p.i. were used to measure VN antibody against the S95 strain. As shown in Fig. 6A, all of the chickens infected with rC-78Rep/S95S and S95E52 developed VN antibody against the S95 strain, with the average VN titer induced by the rC-78Rep/S95S strain ($4^{2.9}$) being comparable to that induced by the S95E52 attenuated strain ($4^{3.4}$). In contrast, sera from chickens infected with the rC-78Rep and C-78E128 attenuated strains obtained in the previous experiment showed very poor cross-neutralization against the S95 strain.

Two SPF chickens were infected with either the virulent S95E4, attenuated S95E52, or rC-78Rep/S95S strain and virus distribution at 7 days p.i. was analyzed by RT-PCR (Fig. 6B). A representative result from the RT-PCR analysis showed that the virulent S95E4 strain was detected in virtually all organs tested, including respiratory, lymphoid, digestive, reproductive, and renal system, as well as in intestinal contents, which are characteristics of a systemic infection with highly pathogenic IBV. In contrast, the S95E52 attenuated strain, like the C-78E128 attenuated strain, was detected only in the upper respiratory tract (larynx and trachea). Interestingly, tissue distribution of rC-78Rep/S95S differed from that of the virulent S95E4 strain as well as the attenuated S95E52 strain. Unlike the restricted distribution of the S95E52 attenuated strain to the upper respiratory tract, rC-78Rep/S95S was detected in larynx/trachea, liver, caecal tonsil, and intestinal contents, but not in other tissues (pancreas, lung, thymus, reproductive organs, kidney, and bursa of Fabricius) that are involved after infection with the virulent S95E4 strain.

3.7. Comparative sequence analysis of the S gene

To better understand the genetic determinants of the diverse IBV phenotypes, including virus pathogenicity, tissue-tropism, and serotype-specific immunogenicity, the S gene sequences of the C-78E6 and S95E4 virulent strains and the C-78E128 and S95E52 attenuated strains were

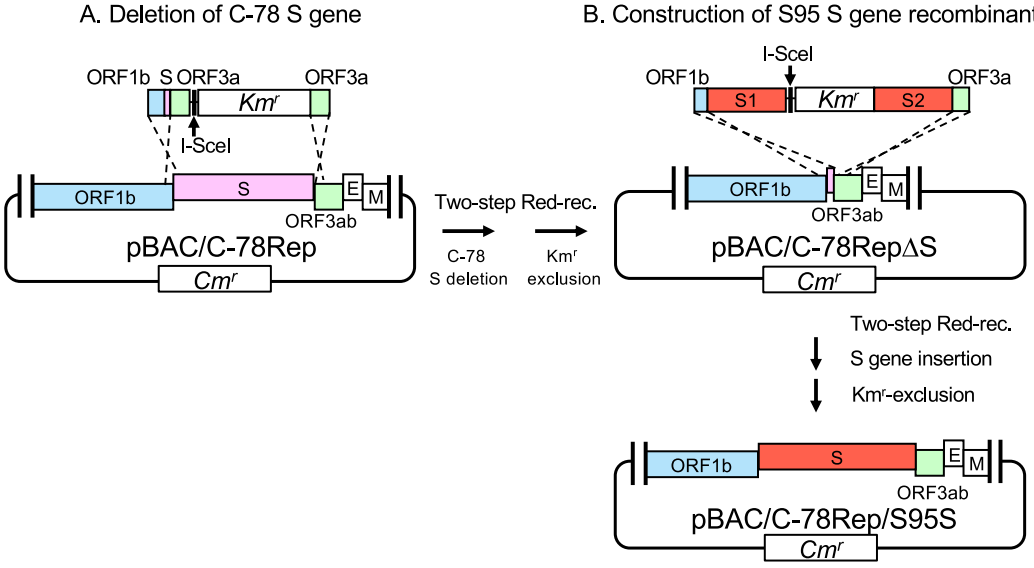


Fig. 5. Deletion of the S gene from pBAC/C-78Rep and construction of S gene recombinant pBAC/C-78Rep/S95S. (A) A transfer gene fragment containing the 3′ ORF1b/5′ ORF3a lacking the S gene, Km^r, and I-SceI site was generated by PCR and homologously recombined into pBAC/C-78Rep using two-step recombination to construct pBAC/C-78RepΔS. (B) Construction of S gene recombinant pBAC/C-78Rep/S95S. The S1 and S2 subunits were separately amplified from S95E4 cDNA and then inserted into pBAC/C-78RepΔS using two-step Red-mediated recombination. The detailed procedure for construction of the S gene recombinant is described in supplementary Fig. S2.

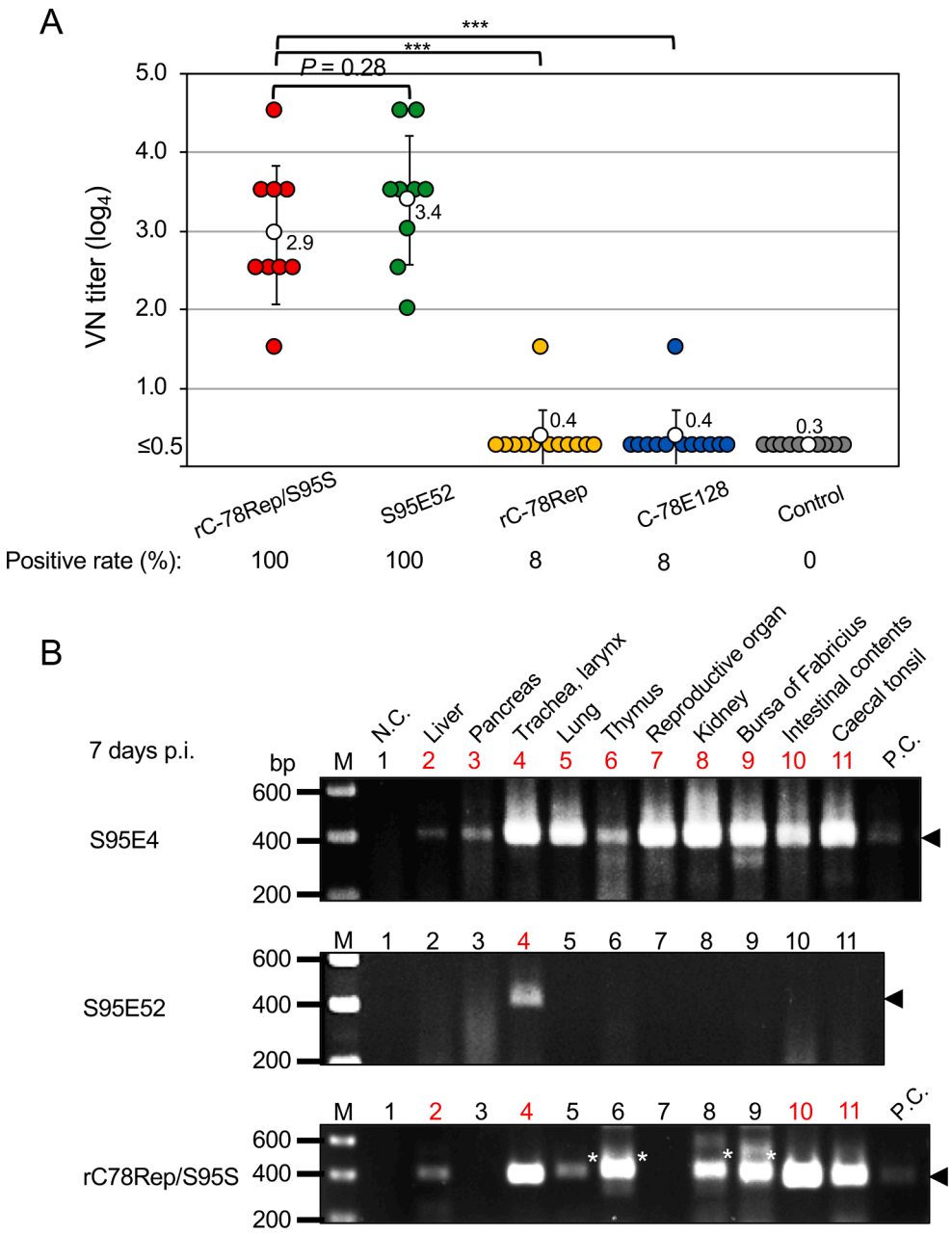


Fig. 6. Immunogenicity and tissue-tropism of virulent S95E4 and attenuated S95E52 strains and rC-78Rep/S95S. (A) Serum neutralizing titers of chickens inoculated with rC-78Rep/S95S or attenuated S95E52 against the S95 strain. The individual neutralization titers and the geometric mean are indicated by colored circles and open circles, respectively. Sera from non-immunized chickens were used as control. The error-bars indicate standard deviation. Statistical significance was determined by Student's *t*-test, and three asterisks indicates $P < 0.001$. (B) Representative RT-PCR results of virus distribution in tissues of chickens infected with virulent S95E4, attenuated S95E52 or rC-78/Rep/S95S at 7 days p. i. are shown. The red numbered lane indicates S2 specific amplification, and the asterisks in the bottom image indicate non-specific amplification. N.C. indicates PBS negative control.

analyzed. Fig. 7 shows amino acid sequence alignment of the viral S proteins along with functional domains and regions identified in previous studies.

The C-78E6 and S95E4 virulent strains had 73 and 51 strain-specific amino acid sequences in the S1 and S2 subunits, respectively, and virtually all of the strain-specific sequences present in the virulent strains were retained in the corresponding C-78E128 and S95E52 attenuated strains. In fact, the S proteins of these attenuated strains had very few, scattered amino acid changes predicted to be associated with virus attenuation, except for a nine amino acid truncation at the C-terminal cytoplasmic tail of the S2 subunit.

Some of the C-78/S95 strain-specific sequences are located in the variable regions of the S1 subunit (VR1, VR2, and VR3 in Fig. 7), which are implicated in contributing to the phenotypic diversity of IBV (Shan et al., 2018). The strain-specific sequences were also found within two recently identified linear VN epitopes (a.a. 87 ~ 93 and 412~428) in the S1 subunit (Zou et al., 2015) and three overlapping VN epitopes (a.a. 669~685, 686~697, and 692~703) in proximity to the N-terminal fusion peptide (FP) in the S2 subunit, corresponding to previous reports (Andoh et al., 2018) (Fig. 7).

IBV infects epithelial cells of the trachea in an alpha-2,3-linked sialic acid-dependent manner (Promkuntod et al., 2014), and the trachea binding domain (TBD in Fig. 7) has been mapped within the a.a. 19 to 69 region that overlaps with VR1 of the S1 subunit (Wickramasinghe et al., 2014). In the TBD, there were seven C-78E6/S95E4 strain-specific amino acids, while all viruses used in this study, regardless of their origin and virulence, commonly infect the upper respiratory tract. Some IBV strains, like the virulent C-78E6 and S95E4 strains, not only infect the respiratory tract but also are disseminated to the kidney and other organs. It has been reported that the nephropathogenic IBV QX strain utilizes a ligand-undefined sialylated glycan to bind to the kidney, and a kidney-binding domain (KBD) in the viral S1 subunit has been mapped within the amino acid segment encompassing residues 99 to 159, particularly amino acids 110 to 112, that overlap with VR2 (Fig. 7) (Bouwman et al., 2020). Within the KBD, there were fourteen C-78E6/S95E4 strain-specific sequences, including the non-conservative

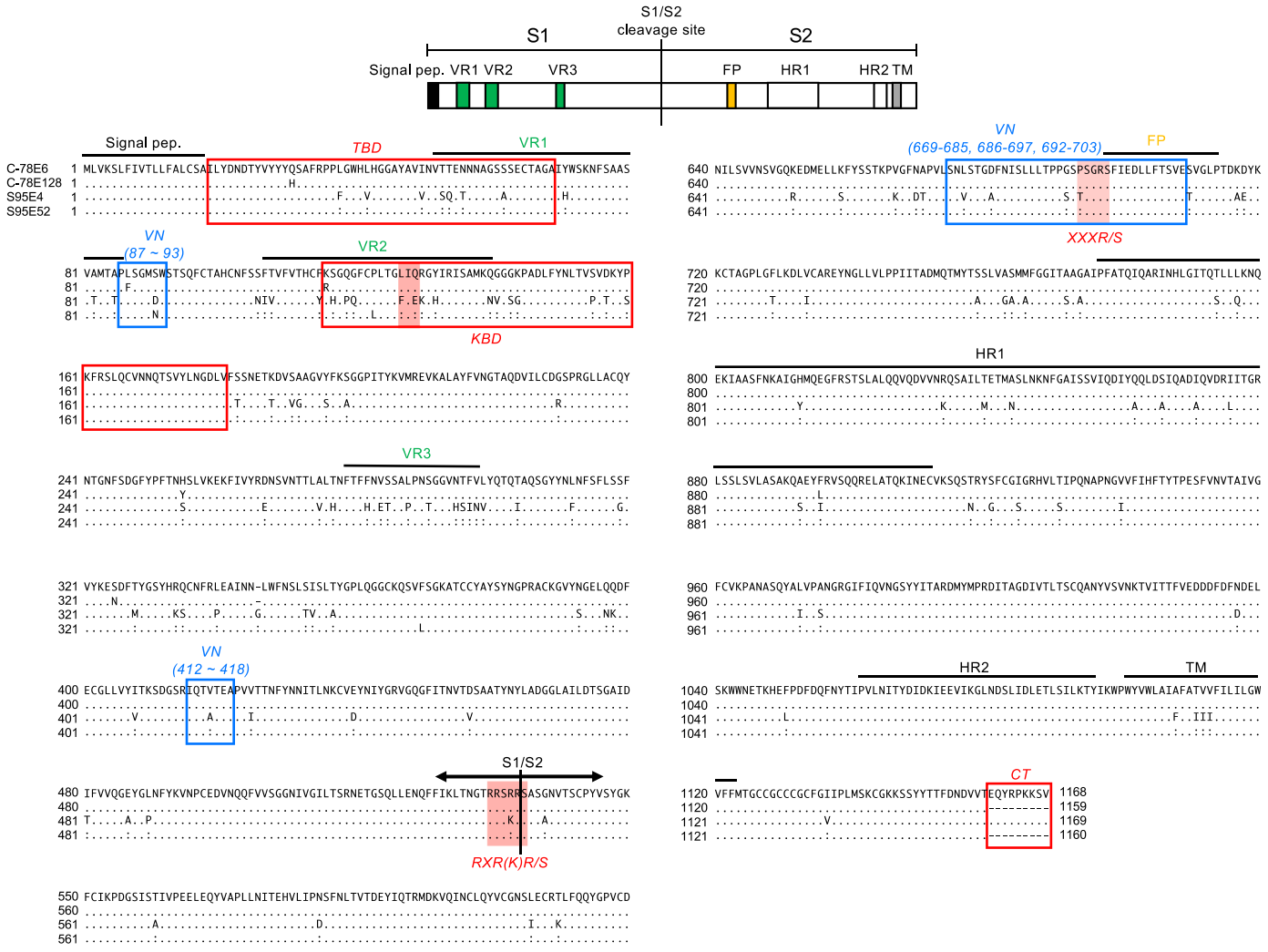


Fig. 7. Alignment of S protein amino acid sequences of the C-78E6, C-78E128, S95E4, and S95E52 strains. Amino acids identical to S protein from C-78E6 are indicated by dots, and colons indicate residues from S95E52 S protein that are identical to S protein from S95E4. Functional domains and regions in the IBV S protein shown in the figure are: variable regions (VR1, VR2, VR3), trachea binding domain (TBD) and kidney-binding domain (KBD) in the S1 subunit, and linear VN epitopes in the S1 and S2 subunits, S1/S2 furin-cleavage site (S1/S2), and an additional S2 cleavage motif (XXX/R), fusion peptide (FP), heptad repeats 1 and 2 (HR1 and HR2), transmembrane domain (TM) and cytoplasmic tail truncation (CT) in the S2 subunit. CT indicates cytoplasmic tail truncation in the S2 subunit of the C-78E128 and S95E52 attenuated strains.

changes of L/F and Q/E at residues 110 and 112, respectively, corresponding to amino acid positions in the S1 subunit of the IBV QX strain.

The cleavage site of IBV S protein into S1 and S2 subunits (S1/S2 in Fig. 7) at a furin consensus motif RXR(K)R/S (X, any amino acid; /, cleavage) (Cavanagh et al., 1986) was conserved in all IBV strains analyzed. Recently, an additional protease cleavage XXXR/S motif, which is conserved in the S2 subunit of IBV and another coronavirus, was identified in proximity to the N-terminal FP (Yamada and Liu, 2009). For both C-78E6 and C-78E128 strains, a P residue occupied the first amino acid position in the XXXR/S cleavage motif, while for S95E4 and S95E52 strains, it was a T residue (Fig. 7).

4. Discussion

In this study, we established a BAC-based RGS for IBV by cloning of the full-length cDNA of the C-78E128 attenuated strain and modification of the virus genome using Red-mediated homologous recombination in *E. coli*. We successfully generated rC-78Rep virus, which represents a virtually identical biological phenotype to the parental C-78E128 attenuated strain. The rC-78Rep/S95S cloned virus, generated by swapping the S gene of the virulent S95E4 strain with that of the avirulent rC-78Rep strain, exhibited an avirulent phenotype and S95 serotype-specific VN immunogenicity, and is therefore expected to be used as an S gene recombinant vaccine.

The BAC-cloned rC-78Rep that was fully competent to induce VN antibody against the homologous C-78 strain was constructed via the partial repair of sequence mismatches present in the ORF1ab gene of the initial rC-78FL construct. We speculate that the mismatch repair in the ORF1ab gene, which regulates viral polyprotein synthesis and processing, promoted surface expression of S protein in virus-infected cells and virus particles and hence increased viral immunogenicity *in vivo*. Previous studies have postulated the importance of ORF1ab sequences in determining IBV pathogenicity (Sommer et al., 2009). In this study, rC-78Rep/S95S containing the S gene of the virulent S95E4 strain in the rC-78Rep backbone was found to be avirulent, implying that the IBV genome(s) other than the S gene determine IBV pathogenicity. Preliminary studies of a BAC-cloned recombinant virus consisting of the 5′-UTR/ORF1ab gene of the C-78E128 attenuated strain and the C-terminal one-third genome of the S95E4 virulent strain from the S gene through to the 3′-UTR indicate that the 5′-UTR/ORF1ab gene determines the attenuated phenotype of C-78E128 (data not shown). The ORF1ab gene is relatively well conserved among IBV isolates, and no correlation

has been found between the ORF1ab genotype and IBV pathotype. Further studies will be needed to identify the genetic determinant(s) of IBV pathogenicity.

Systemic virus dissemination of the C-78E6 and S95E4 virulent strains was in contrast to the upper respiratory tract-restricted infection seen with the attenuated C-78E128 and S95E52 strains as well as the avirulent rC-78FL and rC-78Rep cloned viruses, which have nearly identical S protein amino acid sequences as their virulent strain counterparts. In addition, the systemic infection of the virulent S95E4 to all organs examined could not be fully recapitulated by the rC-78Rep/S95S S gene recombinant virus. These results imply that in addition to receptor usage determined by the viral S gene, other viral factor(s), including viral replicative potential or virulence, are involved in determining IBV tissue tropism *in vivo*.

Sequence analysis of the S protein identified strain-specific sequences in the C-78E6 and S95E4 virulent strains that are strongly conserved in the corresponding C-78E128 and S95E52 attenuated strains, respectively. Except for the cytoplasmic tail truncation in the S2 subunit, the S proteins in the C-78E128 and S95E52 attenuated strains showed only scattered changes associated with virus attenuation. This implies that the attenuation of virulent IBV strains by serial passage in embryonated chicken eggs is not associated with strong selective pressure on the viral S gene, and the functionality of the S protein in the virulent strains is well-retained in the corresponding attenuated strains. Cytoplasmic truncation of the S2 subunit, which is also seen in some laboratory strains of IBV and other enveloped viruses, may be the result of replicative adaptation of primary isolates to embryonated chicken eggs or *in vitro* cell culture, as previously reported (Binns et al., 1986).

The induction of VN antibody against the S95 strain by rC-78Rep/S95S recombinant virus further supports the importance of amino acid sequences in the S protein in determining IBV serotype. As previously suggested (Andoh et al., 2018; Shan et al., 2018; Zou et al., 2015), C-78/S95 strain-specific sequences in the three variable regions (VR1, VR2 and VR3) of the S1 subunit and five linear VN epitopes (a.a. 87 ~ 93 and 412 ~ 418 in S1 and a.a. 669~685, 686~697, 692~703 in S2) may contribute to determine their serotype-specific immunogenicity.

Assessment of tissue distribution of the C-78E6 and S95E4 virulent strains showed systemic virus infection of respiratory, renal, lymphoid, and intestinal organs. The C-78E6/S95E4 strain-specific sequences within the receptor binding domains of the trachea (TBD) and kidney (KBD) shown in Fig. 7 may differently regulate alternative receptor usage of the viral S1 subunit to facilitate systemic virus infection. The C-78E6 and S95E4 strains also had strain-specific P and T residues, respectively, at the first amino acid position of the XXXR/S cleavage motif in the S2 subunit. The single amino acid difference in the XXXR/S motif, which may affect the cleavage of the S2 subunit in a cell type- and protease-specific manner, potentially influences viral cell/tissue tropism, as previously reported in the Vero cells-adapted IBV Beaudette strain (Bickerton et al., 2018).

One of the objectives of this study was to establish a practically feasible genetic platform for developing attenuated vaccines against new IBV variants. By using the virulent S95E4 field isolate, we have provided proof-of-concept for the development of a live attenuated vaccine by swapping the viral S gene into the pBAC/C-78Rep vaccine platform. Direct cloning of the S gene of the IBV field strain into the vaccine platform is advantageous over live attenuated vaccine, inactivated vaccine and recombinant S protein in terms of conservation of the naïve S protein conformation. Adaptive undesired IBV mutations in embryonated chicken eggs or cell culture, disruption of S protein conformation by virus inactivation, and lack of or altered N-linked glycosylation of recombinant S protein all significantly influence S protein immunogenicity, particularly conformation-dependent epitopes of serotype-specific VN antibodies. The direct S gene cloning of IBV variants into a BAC-based vaccine platform represents a straightforward approach to develop a recombinant vaccine against new serotypic IBV variants.

Funding

This work was partially supported by JSPS KAKENHI [Grant No. 25850193].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank Kazumoto Shibuya for his support in progressing this work and Atsushi Kato, Natsumi Takeyama, and Shizuka Yano for helpful comments on the manuscript. We would also like to thank Kazuo Nishigaki for 293T cells, Gregory A. Smith for *E. coli* GS1783, and Nikolaus Osterrieder for pEPkan-S.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2022.100155.

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