JCM Accepts, published online ahead of print on 6 August 2008
J. Clin. Microbiol. doi:10.1128/JCM.00136-08
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Two different PVL phage lineages predominate in Japan

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Abstract

We determined the entire nucleotide sequence of ϕSa2958-carrying Panton Valentine leukocidine (PVL) gene, which was lysogenized in a ST5 type II SCC*mec* strain of methicillin-resistant *Staphylococcus aureus* (MRSA). Based on the nucleotide sequence of PVL phages, we developed polymerase chain reactions (PCRs) to discriminate 5 PVL phages to classify them first into 2 morphological groups (elongate-head type and icosahedral-head type) with 4 PCRs including 2 PCRs for identifying the gene lineage between *lukS*-PV and the tail gene. The phages were classified into 5 types by 4 PCRs identifying each phage-specific structure. With these PCRs, we examined the PVL phage types of 67 MRSA strains isolated in Japan from 1979 through 1985 and since 2000, and found that two morphologically distinct phages were predominant in Japan. The icosahedral-head-type phage, represented by ϕ108PVL type, was identified 39 of 53 strains isolated from 1979 through 1985. Of the other 25 of 26, 25 are mostly belonged to elongated head type: 3, ϕSa2958 type; 8, elongated -head type; 14, and ϕSa2958-like phage of unknown type.

We induced prophages by treatment with mitomycin C from 6 strains of the ϕSa2958 type or ϕSa2958-like unknown type phages; 5 of 6 strains carried intact PVL-carrying phages, which can infect to other *S. aureus* strains and might generate novel-PVL positive strains of *S. aureus*.

That various SCC*mec* elements were carried by different strains of the same phage type,

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suggests that *S. aureus* strains might independently acquire PVL phages before they acquire various SCC*mec* elements.

Introduction

Since the early 1990s, the numbers of strains of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from community-acquired (CA) infections have increased (5, 11, 32). Characteristics of CA-MRSA strains have been investigated and compared with those of healthcare-associated (HA)-MRSA strains. The genotypes and SCC*mec* types of CA-MRSA strains differed greatly from those of HA-MRSA strains. In addition, most of CA-MRSA strains are reported to carry the Panton-Valentine leukocidine (PVL) gene with high incidence (11).

The PVL was first reported in 1932 (36). It is a two-component toxin composed of LukF-PV with a molecular mass of 34 kDa and LukS-PV with a molecular mass of 32 kDa (37). Two proteins, LukF-PV and LukS-PV, act synergistically and cause damage on cell membranes by forming pores, resulting in lysis of polymorphonuclear leukocytes (PMNs) and macrophages (6, 13).

The genes of *LukS-PV* and *LukF-PV* are encoded by prophages that are integrated into the *S. aureus* chromosome. To our knowledge, 6 PVL phages, ϕPV83, ϕPVL, ϕSLT, ϕSa2mw, ϕ108PVL, and ϕSa2usa have been reported to date (1, 9, 21, 22, 29, 33). These phages carry the

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lukS-PV and *lukF*-PV genes that showed more than 99 % nucleotide identity and are integrated at the same position in the *S. aureus* chromosome. However, the morphologies of the phage particles are not identical. The phage morphologies can be classified into two types, the icosahedral-head type and the elongated-head type (4). We have examined PVL-positive MRSA strains isolated from 1979 through 1985 and have determined the nucleotide sequence of ϕ108PVL carried by a type-IV.3-ST30 MRSA strain 81/108 (29). When we have examined the carriage of ϕ108PVL phage in representative MRSA strains isolated from 1979 through 1985, the phage ϕ108PVL was most often identified in ST30 MRSA strains, whereas the ST5-SCC*mec* type II.1 MRSA strain JCSC2958 was negative with the polymerase chain reaction (PCR) in 5 of 6 sets of primers to identify ϕ108PVL, indicating that the strain might carry other new PVL phage. Both PVL-positive methicillin-sensitive *S. aureus* (MSSA) strains and MRSA strains have been isolated worldwide, e.g., in the United States (2, 3, 10, 38), France (41), Australia (7), England (15), Canada (31), Singapore (16), Belgium (8) and Uruguay (28). We wondered how these PVL-positive strains evolved by acquiring each PVL phage and how each PVL phage evolved by acquiring the *luk*S-F-PV genes. In an attempt to answer these questions, we have determined the nucleotide sequence of a new PVL phage carried by JCSC2958 and developed a PCR system to identify the lysogenized PVL phages. By applying the newly developed PCRs, we have found that Japanese PVL-positive *S. aureus* strains carry

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two morphologically distinct PVL phages, the icosahedral head type, represented by ϕ108PVL phage and the elongated head type represented by ϕSa2958, which differ from those disseminated in the United States or France.

Materials and Methods

MRSA and MSSA strains

Sixty-five PVL-positive MRSA strains isolated in Japan were tested. Fifty-three strains were isolated from 1979 through1985: 12 at Tokyo University Hospital; 23 at Gunma University Hospital; 10 at Tokyo Geriatric Hospital; and 9 at The Jikei University Hospital. Twelve PVL-positive MRSA strains were isolated in the past 8 years: a strain isolated from pus on the skin of an outpatient at Juntendo University Hospital in 2002; a strain isolated from an inpatient with pneumonia of Moji Rosai Hospital (identified by the surveillance of PVL-positive strains conducted by the SRL Laboratory and kindly provided by Dr. Hiroshi Kuramoto; a strain isolated in 2007 by Masato Higashiide from a seven year-old boy with an abdominal wound; 2 strains (kindly provided by the LVFX Surveillance Group head by Keizo Yamaguchi) isolated from the pus of a 27-year-old man in 2002 (strain EB00449) and from the centesis of a 61-year-old man in 2000 (strain DB00319) (42); 7 strains isolated by Atsuo Katai at Kinan General Hospital in 2005 through 2007; Strains MW2 and 81/108 were used as representative

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PVL-positive MRSA strains, a strain ATCC49775 was used as a representative PVL-positive MSSA strain; and strain RN4220/ϕSLT was also used as a control for the induction experiment of PVL-positive phages.

To identify phages induced from lysogenized bacteria, two MSSA strains, RN4220 and 1039, were used as indicator strains. These mutant strains that lack prophages and restriction systems, were obtained from the NCTC8325 and Terashima strains, respectively (43).

SCC*mec* typing and identification of virulence genes

SCC*mec* typing and identification of virulence genes were performed with PCRs as described previously (23, 28, 35).

Determination of the entire nucleotide sequence of ϕSa2958

The Fosmid library from the genomic DNA of JCSC2958 was constructed by using the CopyControl™ Fosmid Library Production Kit (EPICENTRE Biotechnologies, Madison, WI, USA) according to the protocols recommended by manufacturer. Briefly, genomic DNA was prepared from cells of JCSC2958 cells with ISOPLANT (Nippon Gene Co., Tokyo, Japan). Approximately 400 μg of the genomic DNAs was sheared into small fragments by aspirating and expelling it through an injection needle. Subsequent size selection of 40 kb-DNA fragments was performed by running samples in agarose gel (Seakem Gold Agarose, Cambrex Bio Science Rockland, Inc. Rockland, ME USA). To recover the size-fractionated DNA after

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electrophoresis, agar slices containing the corresponding sizes of DNA was cut and then melted in GElase solution. The recovered DNA was purified and treated with the End-Repair Enzyme Mix that came with the kit. The purified insert DNA was ligated into CopyControl pCC1FOS Cloning-Ready Vector with DNA ligase, then packaged according to the manufacturer's instructions. Each 10 μl of the Fosmid library was mixed with 100 μl of EPI300-T1R cells and incubated for 20 minutes to promote phage infection. Each reaction mixture was spread on an L-agar plate containing 12.5 μg/ml of chloramphenicol and incubated at 37°C for over night. Five hundred colonies were screened with 2 primer's pairs for the PVL gene and the integrase gene (Table 1), which are conserved in nearly all extant PVL phages. Plasmids were extracted from positive clones and used as templates for nucleotide sequencing. In some cases, DNA fragments amplified by long-range PCR were used as the templates. Primers used for long-range PCR are listed in Table1. Nucleotide sequences were subsequently determined with the primer walking method.

Multi-locus sequence typing and Coagulase typing

Genotypes of representative strains were determined by means of multilocus sequence typing (MLST) with the method of Enright et al (12). Coagulase types of all tested strains were determined with the serological method established by Ushioda et al (39).

Induction of prophages from *S. aureus* cells

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A 0.3-ml-portion of overnight culture was inoculated to 3 ml of BHI broth. After the culture had been shaken for 2 hrs at 37°C, mitomycin C (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) was added to a final concentration of 1 μg/ml, and the culture was cultivated at 37°C with shaking for 50 minutes. Cells were then precipitated by centrifugation and resuspended with 300 μl of L-broth. A 100-μl portion was added to 3 ml of L-broth and incubated further at 37°C until the cell suspension became transparent. The supernatant was sterilized by means of a membrane filter with 0.45-μm-diameter pores (Whatman Corp., Clifton, NJ, USA).

Identification of PVL-carrying phages by plaque hybridization

Phage solutions were diluted by 10-fold serially, and a 0.1-ml portion of each solution was mixed with a 0.3-ml overnight culture of the RN4220 or 1039 strain, and kept at room temperature for 15 min. Three milliliters of L-broth containing 0.6 % agar was added to each mixture, which was then poured onto Heart Infusion agar plates. The plates were incubated over night at 30℃ to form plaques on the lawns. Plates with an appropriate numbers of plaques were selected, and the plaques were transferred onto a piece of Biodyne membrane (Pall Biodyne A; pore size, 1.2 μm; Pall Life Sciences, Ann Arbor, MI, USA). The filters were denatured by submersion for 5 min in 0.5 M NaOH and 1.5 M NaCl, and then neutralized by submersion twice for 5 min in a solution of 0.5 M Tris HCl (pH 7.3), 1.5 M NaCl, and 1 mM EDTA.

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The DNAs were cross-linked to the nylon membrane using the Stratalinker UV Crosslinker (Stratagene Japan K. K., Tokyo, Japan). Probes to identify of PVL-carrying phages were prepared by labeling DNA fragments with digoxigenin using the DIG DNA Labeling and Detection Kit (Roche Applied Science, Penzberg, Germany). The DNA fragments used for plaque hybridization were amplified with PCR and genomic DNA of the JCSC 2958 strain as a template with a pair of primers to identify the *luk*S, F-PV genes (Table1). The subsequent experiment was performed as described by the manufacturer.

PCRs to classify PVL phages

We have developed 8 PCRs to classify the PVL-carrying prophages using chromosomal DNA preparations from PVL-positive *S. aureus* strains as templates. Primers used for identifying PVL-carrying phages are listed in Table 1. These primers were designed on the bases of nucleotide sequences of 5 PVL phages: ϕ108PVL, ϕSa2mw, ϕPVL, ϕSLT, and ϕSa2958 deposited in DDBJ/EMBL/GenBank databases under accession nos. AB009866, BA000033, AB009866, NC_002661, and AP009363, respectively. The locations of the primers are shown in Figure 2.

Two PCRs were designed to identify the carriage of 2 morphologically distinct phages. PCR-1 was designed to identify the carriage of phages with isometric hexagonal heads by amplifying the portal gene and the head gene, which are conserved in ϕPVL and ϕ108PVL.

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PCR-2 was designed to identify the carriage of phages with elongated heads by amplifying the portal gene and the head gene, which are conserved in ϕSa2mw, ϕSa2958, and ϕSLT. If 2 DNA fragments could be amplified with PCR-1, we then proceeded to PCR-3, which was designed to identify the gene lineage between the tail gene and *lukS* gene and verify that the tail gene identified with PCR-1 belonged to a PVL-carrying phage with a hexagonal head. If 2 DNA fragments were amplified with PCR-2, we proceed to PCR-4, which was designed to identify the gene lineage between the tail gene and *lukS* gene to verify that the tail gene of the elongated shape is located in relation to *lukS* gene with a primer pair commonly conserved among ϕSa2958, ϕSa2mw and ϕSLT.

PCRs 5 to 8 were designed to identify 5 PVL phages by amplifying the gene lineage between the integrase gene, which is commonly carried by all reported PVL-carrying phages, and genes located on the region related to lisogeny or recombination of each PVL phage.

PCR-5 was designed to identify ϕPVL and ϕ108PVL by integrase and two ORFs, *repressor* for ϕPVL and *antirepressor* for ϕ108PVL. PCR-6, 7, and 8 were designed to identify ϕSa2958, ϕSa2mw, and ϕSLT by detecting the gene lineage between the integrase gene and the genes located at the downstream of the gene: a hypothetical protein (JCP004) for ϕSa2958, cro repressor protein for ϕSa2mw, and a single-stranded binding protein for ϕSLT.

Chromosomal DNAs were prepared with the small-scale phenol extraction method and used as

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PCR templates. The PCR reactions were performed using a thermal cycler Gene Amp 9600 (Perkin-Elmer Cetus Instruments, Emeryville, CA., USA). The reaction mixture for PCR-1, 2, 5-8 contained 50 ng of template DNA, each oligonucleotide primer (0.2 mM), 400 mM of each dNTP, 1x Ex Taq buffer with magnesium and 4U of Ex Taq polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan) in a final volume of 50 μl. PCR reactions for 5 PCRs (1, 2, 5, 6 and 7) consisted of 30 cycles of denaturation (95°C, 60 sec), annealing (50°C, 60 sec) and extension (72°C, 2 min).

PCR-3, 4 and 8 were carried out with long-range PCR using Expand High Fidelity PCR kit following to the protocols recommended by manufacturer. The six μl of PCR reaction mixture was subjected to agarose gel electrophoresis to detect amplified DNA fragments.

Nucleotide sequence accession number

The entire nucleotide sequence of ϕSa2958 has been deposited in the DDBJ/EMBL/GenBank database under accession no. AP009363.

Results

The structure of a novel PVL phage, ϕSa2958

The ϕSa2958 phage is 46046 bp in length, which is comparable to other PVL phages identified to date. A total of 59 predicted open reading frames (ORFs) larger than 99 bp were identified in

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ϕSa2958 (Fig.1 and Table 1). The whole G+C content of the novel PVL phage was 33.1 %, which is comparable to those of *S. aureus* genomes. The gene coding potentials for ϕSa2958 was 90.6% with approximately 1.28 genes/kbp of nucleotide sequence.

The 29-bp core sequence in ϕSa2958 exactly matched the corresponding core sequence conserved in ϕSa2mw, but differs from those of ϕSLT, ϕPVL and ϕ108PVL by 1 base pair. The 25-bp sequences of the attB site located on the chromosome side were identified flanking to both ends of ϕSa2958. The right-most (attB-R) sequence was identical with that of ϕSa2mw, whereas, the left most (attB-L) sequence differed by 2 base pairs from that of ϕSa2mw and differed significantly from those of ϕPVL and ϕ108PVL. The 25-bp attachment sites (attP) at both ends of the phage were well conserved among all 5 PVL phages.

The organization of the ϕSa2958 genome was similar to that of extant PVL phages, indicating the order of modules on the phage genome is well conserved: regions related to lysogeny, DNA replication/transcriptional regulation, the packaging/head, the tail and lysis module as well as *LukS, F*-PV. Fifty-nine ORFs were roughly classified into 5 groups on the basis of similarities with extant PVL phages. Group-1 contained 10 genes (JP013, JP014, JP023, JP024, JP027, JP056, JP057, JP058, JP059, and JP060) conserved among 5 extant PVL phages with nucleotide identities of more than 90% (dark green ORFs in Figure 1). The integrase gene (*int*) was located at the left-most side of ϕSa2958. It showed high similarity to those carried by

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extant PVL phages with more than 98% identity in amino acids. ORFs JP056, JP057, JP059, and JP060 encoded holin, amidase, LukS-PV and LukF-PV proteins, respectively. Both the *LukF-PV* and *LukS-PV* genes showed nucleotide identities of more than 99 % with those of other PVL phages. In particular, the *loo*k-PV gene in ϕSa2958 exhibited 100% homology with that of ϕPVL. ORF JP058 encodes 48 amino acids with no assigned function identified by means of a BLASTP search. This ORF was also identified in ϕPVL, although it was disrupted and could not be assigned because of the insertion of a 'T' nucleotide. Group 2 contained 27 orbs (JP011, JP012, JP028 and JP032-55) conserved among ϕSa2958, ϕSa2mw and ϕSLT (light green in Fig1). Because 8 orbs that might be associated with phage DNA packaging and head and tail formation are involved (JP034 [terminate small subunit], JP035 [terminate large subunit], JP006 [portal protein], JP007 [preheat protease], JP008 [cased protein], JP043 and JP044 [major tail protein], and JP047 [phage tail tape measure protein]), ϕSa2958 could be determined on the basis of taxonomy criteria to belong to the same subgroup as ϕSa2mw and ϕSLT.

Group 3 contained 12 orbs (JP015-JP022, JP025, JP029, JP030 and JP031) that showed high similarity with only ϕSa2mw (pink bars in Fig. 1). Three genes were associated with phage replication during the phage lyric process. Representative genes grouping group 3 encode DNA polymerase (JP019), DNA-binding protein (JP021), and helices (JP031), and

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235 virulence-associated protein E (JP029). Group 4 contained 3 ORFs that were homologous to
236 other only 1 or 2 other phages. JP026 (yellow), JP003 (orange), and JP010 (blue) were
237 homologous only to ϕ108PVL and ϕSLT, to ϕPVL and ϕSLT, ϕ108PVL, respectively.

238 Group 5 contained 6 ORFs (JP004-9) that were uniquely conserved in ϕSa2958 (no color in
239 Fig. 1). Interestingly, JP006, encoding the transcription repressor gene was not homologous to
240 the corresponding gene in other extant PVL phages. A search with the BLASTP program
241 revealed that JP006 showed 100% identity with the corresponding gene conserved in lytic
242 phage 47 and showed 65% identity with the lytic phage 29, whose entire genomic sequences
243 have been determined by Kwan et al (24).

244 The JP006 protein contains a helix-turn-helix DNA binding motif that is present in a large
245 family of transcriptional regulators. Another unique ORF, JP008, conserved in ϕSa2958, which
246 was identified as an anti-repressor gene, was shown to have 93% similarity with the
247 corresponding gene in phage 47. The remaining 4 unique ORFs in ϕSa2958 could not be
248 assigned any functions through searches with the BLASTP program.

249

250 **Characterization of PVL-carrying prophages in Japanese MRSA strains**

251 We conducted PCR experiments to identify PVL phages carried by 53 MRSA strains isolated
252 in from 1979 through 1985 and 12 MRSA strains isolated in the 2000s with PCRs as described

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253 in the Methods section. The representative results of these PCRs are shown in Figure 3 and the
254 results of PCR experiment are summarized in Table 3. When PCR-1 was performed with
255 chromosomal DNAs of 5 strains carrying each PVL phage, DNA fragments of 2 expected sizes,
256 489 bp for the tail gene and 569 bp for the head gene, were amplified with chromosomal DNAs
257 of 81/108 (ϕ108PVL), ATCC49775 (ϕPVL), and MW2 (ϕSa2mw). When PCR-2 was
258 performed with chromosomal DNAs of 5 strains, expected sizes of 2 DNA fragments, 466 bp
259 for the tail gene and 655 bp for the head gene, were amplified with chromosomal DNAs of
260 JCSC2958 (ϕSa2958), RN4220 (ϕSLT), and MW2 (ϕSa2mw), and 81/108 (ϕ108PVL).
261 MW2 was positive with the PCR-1 and PCR-2, because this *S. aureus* strain carries two
262 additional different phages in its chromosomal DNA, and one of them, ϕSa3mw, has the same
263 morphogenesis region as does ϕPVL (1, 19). Strain 81/108 was also positive with PCR-2 and
264 seemed to carry other phages that did not carry *lukS-F PV* genes and have the structure to react
265 with primers for PCR-2. So far, most of PCR-1-positive strains we have tested have also been
266 PCR-2 positive.
267 Therefore, to confirm whether the structures related to morphology belonged to PVL-carrying
268 phages, we performed PCR-3 and PCR-4 to verify whether the PVL-carrying prophage belonged
269 to either the icosahedral-head-type phage or elongated-head-type phages by amplifying gene
270 lineages between *lukS* and *mtp* that belong to either the icosahedral-head type or the

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271 elongated-head type.

272 DNA fragments of expected sizes were amplified with PCR-3 and chromosomal DNAs of
273 81/108 (ϕ108PVL) and ATCC49775 (ϕPVL), and with PCR-4 and chromosomal DNAs of
274 MW2 (ϕSa2mw), JCSC2958 (ϕSa2958) and RN4220 (ϕSLT).

275 So far, 38 of 39 PCR-1-positive strains have been positive with PCR-2. However, PCR-3 with
276 was performed with chromosomal Danas of 39 strains, and DNA fragment of 10.5 kb was
277 successfully amplified from 35 of 39 PCR-1 positive strains, indicating that these strains carried
278 PVL-carrying phages of the icosahedral-head type. Sixty-six of 67 strains were positive with
279 PCR-2, whereas, DNA fragments of different sizes were amplified with chromosomal DNA of
280 the JCSC4274. When we performed PCR-4 with chromosomal DNAs, 26 strains in which no
281 DNA fragment or 1 DNA fragments was amplified, DNA fragments were amplified with
282 chromosomal DNAs of 11 of 26 strains.

283 Furthermore, we have performed PCRs 5 to 8 to classify them into 5 types of PVL phages. We
284 have conducted PCR-5 to determine whether these phages belonged to either the ϕPVL type or
285 the ϕ108PVL type. A DNA fragment of 4340 bp, indicating the carriage of ϕ108PVL-specific
286 gene (fragment PCR-1 A), was amplified with the DNAs of 33 of 35 strains, whereas a DNA
287 fragment of 1411 bp, indicating the carriage of ϕPVL (fragment PCR-2 A), was amplified in 2
288 cases together with a DNA fragment of 4340 bp. Because 2 PCR experiments to identify the

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other component of ϕPVL yielded the negative results and 4 PCR experiments to identify the ϕ108PVL- specific regions yielded the positive results (data not shown), we have concluded that these 33 strains that were positive with PCR-1, amplifying fragment PCR-1 A, which carried the phage belonging ϕ108PVL type. Four strains that were negative with PCR-4 were confirmed to belong to ϕ108PVL type with 4 PCR experiments to identify ϕ108PVL-specific regions and 2 long-range PCRs to amplify the DNA fragments covering the region from *mtp* to l*ukS*. Therefore we regarded these 4 strains as being of the ϕ108PVL-like type. Two strains, with the gene lineage between *lukS* and *mtp* common to ϕ108PVL and ϕPVL, could not be classified into 1 of 5 extant PVL phages so far tested.

In contrast of the 26 strains in which no or one DNA fragment was amplified with PCR-1, 25 were positive with PCR-2. In the case of strain JCSC7247, only 1 DNA fragment, suggesting the carriage of *mtp,* in common with that of the elongated head type was amplified.

PCR-4 was performed with DNAs of 26 strains to verify the gene lineage between the tail gene and the *lukS* gene with a primer pair designed on the tail gene that is commonly conserved in ϕSa2958, ϕSa2mw and ϕSLT, and *lukS*. Of the 14 strains isolated from 1979 through 1985, only 1 was positive with PCR; in contrast, 10 of 20 strains isolated in the 2000s were positive with PCR. In addition, we have performed PCR-6, -7, and -8 to classify elongated-head-type phages as either the ϕSa2958 type, the ϕSa2mw type or the ϕSLT type. Interestingly, neither the

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ϕSa2mw type nor ϕSLT type, which are carried by *S. aureus* strains isolated in the United States or France, was identified, whereas, 3 strains carrying ϕSa2958-type phage and 8 strains carrying the elongated-head-type phage were identified. We have determined the nucleotide sequence of a prophage lysogenized in an MRSA strain, JCSC2958, isolated in Japan in 1981. However, 13 of 14 strains isolated from 1979 through1985 were definitively determined to be of the ϕSa2958 type, because we could not amplify the region between *mtp* and *lukS* with long-range PCR. Because these 13 strains as well as a strain isolated in the 2000s were positive in PCR-6, identifying a specific ORF in ϕSa2958, and in several types of long-range PCR to amplify the gene lineage between *mtp* to some ORFs of ϕSa2958 located between the tail gene and l*ukS*, e.g., JP052 or JP053, we consider them to be ϕSa2958-like phage of unknown type. One strain carried a prophage that could not be classified into either one of the two types, was exceptional, because it was negative with 7 PCRs other than PCR-6.

Characterization of PVL-positive MRSA clones

We next characterized these strains by determining the coagulase isotypes and SCC*mec* types of all 65 strains and the MLST types of selected strains (Table 4). Fifty-two of the 53 MRSA strains isolated from 1979 through 1985 and 11 of the 12 MRSA strains isolated in the 2000s carried type 4 coagulase. Only 1 strain, JCSC2958, that we used for sequencing the ϕ2958PVL genome carried type 2 coagulase and only 1 strain, JCSC7247, carried type 7 coagulase. When

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we performed MLST of the chosen isolates, we found that all coagulase type 4 strains belonged to sequence type (ST) 30, whereas, a coagulase type 2 strain belonged to ST5, and a coagulase type 7 strain belonged to ST59. The data showed that the genotypes of MRSA strains carrying PVL phages isolated in Japan were extremely homogeneous, and were represented by ST30-coagulase type 4 strains.

However, the SCC*mec* elements carried by these strains were extremely diverse. Among the 33 MRSA strains identified as the ϕ108PVL-type, type IV.3 SCC*mec* strains (21 of 33) were the most frequent and were followed by type IV.1 SCC*mec* strains (8 of 33), type II SCC*mec* strains (2 of 33) and type I SCC*mec* strains (1 of 33). Three strains identified as the ϕSa2958 type carried type-II SCC*mec* and type-IV.1 SCC*mec* elements. Eight strains classified as the elongated head type carried type-IV.3 SCC*mec* and nontypeable SCC*mec* elements, which were similar to type-IV.3 because they carried type-2 *ccr* and the J1 region of type-IV.3 SCC*mec* and IS*1272*. Since we could not amplify the gene lineage between *mecA* and IS*1272*, we tentatively considered them as untypeable. Other strains were also carried several SCC*mec* elements.

Interestingly, no isosahedral-phage-type strain was identified in isolates form 2000s. The prophages of three strains were judged to be of the ϕ2958 type. In contrast to JCSC2958, another two strains isolated in the 2000s belonged to the genotype coagulase type 4-ST30 and carried a type-IV.3 SCC*mec* element. A ϕSa2958-like phage of unknown type strain was

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identified in the 2000s as belonging to the coagulase type 4-ST765 (CC30) and carried type I SCC*mec* and was similar to the isolates from 1979 through 1985. A strain carrying an untypeable PVL-carrying phage belonged to coagulase type 7-ST59 and carried the type-V SCC*mec* element.

Induction of infective PVL phages

To date, the ϕSLT is the only PVL phage that has been induced from cells and can infect other cells. Through a structural comparison of PVL phages, we found that both ϕSa2958 and ϕ Sa2mw carried possibly intact genes encoding head and tail proteins, and we inferred that these PVL phages might also be infective. To confirm this hypothesis, we have induced prophages by treating these phages with mitomycin C and mixed them with cells of restriction-negative strains, RN4220 and 1039. Because plaque formation does not indicate whether theses plaques were generated by induced PVL phages, we conducted plaque hybridization experiments with a probe for the *luk*S-F-PV genes to examine whether these plaques were of PVL-carrying phages. The results are summarized in Table 5. We tested 4 MRSA strains (JCSC2973, JCSC4465, M11 and JCSC2958) isolated from 1979 through1985 and MRSA strains isolated in 2002 (JCSC4625) and in 2004 (JCSC6605). Three strains carried ϕSa2958-type PVL phages, and 3 strains carried ϕSa2958-like-type PVL phage . In addition, prophages, ϕSa2mw and ϕ108PVL,

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whose genomes have been sequenced, were induced from strains MW2 and 81/108 as well as ϕSLT from which lysogen, RN4220 (ϕSLT). The cell suspension of 8 of 9 tested strains became transparent after the addition of mitomycin C, indicating that phages might be generated upon the stress of DNA synthesis inhibition by mitomycin C. When these phage lysates were diluted and mixed with cells of indicator strains RN4220 and 1039, we found most of the induced phages could propagate in 1039, whereas only some of them could propagate in RN4220.

As expected, phage lysates from MW2 and JCSC2958 generated plaques on the 1039 and RN4220 lawn, which hybridized with the probe for the *luk*S-F-PV genes, indicating that the ϕSa2mw and ϕSa2958 can infect to other *S. aureus* strains as can a previously reported ϕSLT. The phage lysate from 81/108 generated plaques, but no PVL-positive plaque was observed, indicating that the ϕ108PVL phage did not induce or cannot infect the strains, tested so far. This result was consistent with our speculation that the ϕ108PVL might be defective, because the tail gene of ϕ108PVL was disrupted in a manner similar to that of ϕPVL (21).

Phage lysates of 5 of 6 MRSA strains carrying either the ϕSa2958-type PVL phage or the ϕSa2958-like-type PVL phage generated PVL-positive plaques on indicator strains; however, the ratio of PVL-positive plaques ranged from 2% to 100 %. In the case of JCSC6055, whose cell suspension did not become transparent after the addition of mitomycin C, no plaques were formed on the lawn of 1039 and RN4220 after the addition of the filtrate.

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Discussion

Characteristics of ϕSa2958

Canchaya has classified the *Staphylococcal* phages into 5 groups based on similarities in genes related to morphogenesis (4). All extant PVL phages are Sfi21-like *cos*-site *siphoviridae*, which can be further subdivided into 2 groups based on their morphology those with isomeric hexagonal head and those with elongated heads. The PVL phages, ϕSa2mw and ϕSa2usa, which are carried by MRSA strains isolated in the United States, have elongated heads. The ϕSLT phage was isolated from a French strain of *S. aureus* and has been shown with electron microscopy to have an elongated head measuring 100 x 50 nm and a flexible tail 400 nm long (33). Interestingly, the ϕSa2958 phage we described here belongs to the elongated-head group, whereas ϕ108PVL, identified in Japan, has an isometric hexagonal head, as does ϕPVL (20).

When we performed a detailed comparison of the genomic structure of ϕSa2958 with those of extant PVL phages, represented by ϕSa2mw and ϕ108PVL (Figure 2 and Table 3), we found that most of the region between ϕSa2958 and ϕSa2mw was conserved. Similarly, this region was also conserved amongϕSa2usa and ϕSLT. In contrast, the regions encoding ORFs related to lysogeny (ORFs JP004-9) were unique to each phage. The average G+C content of region from JP004 to JP009 was approximately 29.2%, which was lower than the average of the whole

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397 ϕSa2958 genome (33.1%). These data indicated that these genes might be acquired from
398 organisms with lower G+C contents.

399 Modular exchange in phages, which is known as the modular theory, has become the basis of a
400 popular hypothesis for phage evolution (27, 34). According to this theory, ϕSa2958 and
401 ϕSa2mw must share a common ancestor and have evolved as close relatives and the module
402 exchange was likely a recent event. We have noted here that functionally important structures of
403 the repressor and antirepressor genes were located in this module (26). We now know that in
404 most known prophages, immunity is elicited by a repressor protein that prevents transcription
405 initiation at promoters controlling expression of lytic function (14, 25). Furthermore, the
406 immunity of the phage is sometimes complicated by the presence of an anti repressor gene that
407 can prevent the expression of the repressor activity. When compared to the nucleotide sequence
408 of repressor genes conserved in ϕSa2958 and ϕSa2mw, they exhibited nucleotide sequences at
409 DNA level distinct from each other; however when we investigated the protein structure
410 encoded by these repressor genes, we found that the essential helix-turn–helix domain was well
411 conserved indicating that both repressor genes are executing functional activities. This finding
412 suggests that ϕSa2958 and ϕSa2mw might utilize different mechanisms for maintaining their
413 immunity when they are integrated in bacterial genomes; to achieve this goal, these phages had
414 to adopt the most beneficial module during the process when they interacted with host bacteria

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or with other phages.

Two lineages of PVL phage are predominate in MRSA and MSSA strains in Japan.

We designed several PCR reactions to identify PVL phages with the notion that the type of PVL-carrying phage should be determined on the bases of the gene lineage between *lukS, F*-PV and other phage components. We first developed PCR-1 and -2 to identify portal or head genes but we soon noticed that these PCRs might also be used to identify components of phages other than PVL-carrying phages. Therefore, we have developed PCRs to identify the gene lineage between *lukS* and *mtp*, which is located rather far from the *lukS-F*-PV genes. In addition, we developed PCRs to classify individual phages by identifying the genes in lysogeny-related regions or recombination-related regions in combination with the integrase gene, which is carried by all reported PVL-carrying phages. During this step, we noticed that some modules are commonly shared among phages. As shown in Fig.2, ϕSa2958 was similar to ϕSa2mw, and ϕSa2958 was similar to ϕSLT (data not shown). The primer pairs we reported here are only selected pairs that react only 1 of 5 phages.

We have found that 2 PVL lineages, the icosahedral head type and the elongated head type, are present in Japan. Phages of the ϕ108PVL type strains were identified mostly in MRSA strains isolated from 1979 through 1985. Of the 53 MRSA strains examined, 33 isolates were identified

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as the carrier of PVL-carrying phage of the ϕ108PVL type, and 4 isolates were identified as carriers of the ϕ108-like type; however, no phages of this type were identified in the isolates from 2000s. Furthermore, no ϕPVL type strain was identified among the MRSA strains tested. However, when 13 PVL-positive MSSA strains isolated in the 2000s were examined, 2 were found to belong to icosahedral-head type, and were likely to be of the ϕPVL type. Because the tail gene of ϕ108PVL was truncated, we assume that the phage had already lost its infectivity but carried an integrated truncated prophage, which might explain why the ϕ108PVL type strain has not been identified recently.

In contrast, phages with an elongated head, e.g., ϕSa2958 as well as ϕSa2mw and ϕSLT, carried intact tail and head genes. ϕSa2958 was identified from an ST5-type II SCC*mec* MRSA strain identified in 1981, whereas, 2 ϕSa2958 type strains of ST30-type IV.3 SCC*mec* strains were identified in the 2000s, suggesting that the phage might infect other strains and become integrated into their chromosomes as a prophage. We considered the prophages carried by 14 strains (13 isolates from 1975 to 1985 and 1 isolate from the 2000s) as ϕSa2958 like phage of unknown type, because we could not clarify the gene lineage between *mtp* and *lukS-PV*. The strains carrying the prophage might be derived from the same clone because they belonged to ST30 and carry type I SCC*mec* element and because the prophages of all 3 tested strains could be induced, proving their infectivity; these findings suggest that the recent isolate might be a

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descendant or that the phage might infect to other strains. Interestingly, 8 strains with elongated -head-type PVL-carrying prophages were identified. Because these strains were identified not only from 7 isolates in Wakayama prefecture, but also from an isolate of a 27-year-old man in Kanagawa prefecture, which is far from Wakayama, in 2002 (strain EB00449), we speculate that the prophage might have been induced from the cells of *S. aureus* and then infected other *S. aureus* cells to generate the novel PVL phage. A coagulase type-7 ST59 strain carried an as yet untypeable PVL phage. Vandenesh et al. have reported that 5 major STs that carry PVL phages, ST1, 8, 30, 59, and 80, and that ST59 PVL-positive strains have been isolated in the United States (40). Because the ST59 strain is not common in Japan, the strains of other countries might have been introduced to Japan. Whether the strain carries a novel PVL phage is the next problem that should be clarified. It might carry a PVL phage that does not belong to the 2 extant groups.

When we characterized these PVL-positive strains by means of SCC*mec* typing and genotyping, we found that the most of them produced type-IV coagulase and belonged to ST 30 or CC30 and carried variable types of SCC*mec* elements including type I, type II, type IV.1 and type IV.3 (17, 18, 30). We speculate that MSSA of the coagulase type 4-ST30 independently acquired 2 phages, ϕ108PVL and ϕSa2958 or other elongated-head phages, before they acquired SCC*mec* elements. The characterization of PVL phages carried by MSSA strains

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isolated in the 1960s has shown that MSSA strains carrying either of the PVL phages were present in the Japanese community. Therefore, we presume that the MSSA strains evolved into PVL-positive MRSA strains by acquiring SCC*mec* elements of various types. When we examined the types of SCC*mec* elements carried by PVL-positive MRSA strains, we found that 20 of 33 MRSA strains of the ϕ108PVL type carried type IV.3 SCC*mec* and that 8 of 33 carried type IV.1 SCC*mec*. It was noteworthy that most of the PVL-positive type-IV.1 SCC*mec* strains had been isolated at the Tokyo Geriatric Hospital, whereas, and most of the PVL-positive type-IV.3 SCC*mec* strains had been isolated at Gunma University Hospital. The data indicated that MRSA clones disseminating in different Japanese hospitals were not identical and that the spread of MRSA strains is regional.

The ϕSa2958 and ϕSa2958-like phages of unknown type are intact prophages

When we studied the structure of the ϕSa2958 identified in this study, we realized that the amino acid sequence of the tail-length tape measure protein designated as JP047 showed 98% similarity in with that of ϕSLT, which has been shown to be the first PVL phage that could infect *S. aureus* strains experimentally (33). Because the tail-length tape measure protein of ϕSa2mw carried by MW2, a CA-MRSA isolated in the American state of North Dakota, was similar to that of ϕSLT, we presumed that the newly identified ϕSa2958, as well as ϕSa2mw

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might also be able to infect to *S. aureus* strains.

To determine whether these 2 phages, as well as the phages of ϕSa2958-type, were intact and could infect *S. aureus* strains, we tested MW2 and 6 MRSA strains carrying ϕSa2958 type- or ϕSa2958-like PVL phage of unknown type. The PVL phages carried by 5 of 6 MRSA strains could infect indicator cells, although the ratios of PVL phages in the phage lysate were not similar. In addition, the sizes of plaques generated on the lawn were larger with 1039 than with RN4220. With 3 strains, M11, JCSC2958, JCSC4625, all phages induced by treatment with mitomycin C were PVL-positive. In contrast, with 2 strains, JCSC2973 and JCSC4625, the ratios of PVL-positive phages were very low. In the case of JCSC6055, which did not generate any plaques, the apparent reason phages were not induced was that the cell suspension did not turn transparent after mitomycin C was added. These discrepancies may be due to many factors of host cells, e.g., the presence of other prophages and differences in gene expression that confer the SOS response. Although we did not test a large number of strains, we have chosen ϕSa2958-type MRSA strains of representative genotype identified in this study. Except for JCSC6055, the 5 strains were derived from 4 different clones, JCSC2973 (ST30-SCC*mec*-I), M11 and JCSC4625 (ST30-SCC*mec*-IV.3), JCSC4465 (ST30-SCC*mec*-IV.n), and JCSC2958 (ST5-SCC*mec*-II), generated intact PVL phages. This result indicates that ϕSa2958 type and ϕSa2958-like phages of unknown type can be frequently induced from many *S. aureus* strains

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with diverse genetic backgrounds. These PVL phages might be induced with or without any stress from *S. aureus* and infect other appropriate recipient *S. aureus* strains, resulting in phage conversion of nonvirulent bacteria to virulent bacteria. The fact that 2 recently isolated strains carried ϕSa2958, and that 1 of them generated infective PVL phages suggests that ϕSa2958 type might be the cause of the recent emergence of PVL-positive MRSA strains in Japan. As shown in Table 5, ϕSa2mw carried by MW2 was infectious for 2 indicator strains, RN4220 and 1039. The USA300 strain has recently been the predominant CA-MRSA clone in the United States. The ϕSa2usa carries PVL and belongs to the same family as ϕSa2mw and ϕSa2958, which have elongated heads. Although we could not identify any plaques of ϕSa2usa, we have tested FPR3757, whose genome has been sequenced; these ϕSa2mw-type or ϕSa2usa-type PVL phages might be the cause of the recent appearance of PVL-positive MRSA strains in the United States. Our data also indicate that ST30 MRSA strains carry two different PVL phages. Further study will clarify whether ST30 PVL-positive MRSA clones can be grouped with Western-Samoan clones.

In conclusion, we have developed a method of combining several PCR reactions to identify PVL phage carried by an organism. Our results indicate that 2 lineages of PVL phages have existed in Japan for many years and that intact phages with elongated heads, such as ϕSa2958 and ϕSa2958-like phage of unknown type or other phages known simply as elongated

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head types, might disseminate among MRSA strains in Japan and confer greater virulence.

Acknowledgements

We thank Dr. Yukio Utsui (Daiichi-Sankyo Pharmaceutical Co. ltd), Dr. Toyoji Okubo (Gunma University), and Dr. Takashi Inamatsu (Tokyo Geriatric Hospital) for providing MRSA strains isolated from 1979 through 1985. We thank Dr. Toyoko Oguri (Juntendo University Hospital) for providing MRSA strain JCSC4625, and Dr. Takashi Kuramoto (Moji Rosai Hospital) for providing MRSA strain JCSC6055. We thank the staffs of SLR Laboratory for conducting PCR experiments to identify PVL-positive *S. aureus* strains. We thank Keizo Yamaguchi (Toho University) for providing MRSA strains collected by the surveillance study for the susceptibility testing for quinolone antibiotics.

This work was supported by a Grant-in-Aid for 21st Century COE Research and a Grant-in-Aid for Scientific Research on Priority Areas and a Grant-in-Aid for Scientific Research C19590456 from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

Legend for figures

Fig. 1.

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(A) The structure of ϕSa2958. Black arrowheads indicate the location of primers used to amplify the entire ϕSa2958 genome. The 2 red arrowheads flanking the core sequence indicate the *att* sites on the phage element.

(B) The ORFs in ϕSa2958. The ORFs are shown as squares in 6 possible reading frames. The direction of the arrows indicates the transcriptional direction for each ORF. Color codes are as follows: dark green, ORFs (or the parts of ORFs) that are well conserved among 4 other PVL-carrying phages, ϕSa2mw, ϕSLT, ϕPVL and ϕ108PVL; light green, ORFs that are highly homologous to ϕSa2mw and ϕSLT; pink, ORFs that are highly homologous to ϕSa2mw; orange, an ORF that is homologous to ϕSLT and ϕPVL; yellow, an ORF that is homologous to ϕSLT and ϕ108PVL; white, ORFs that are unique to ϕSa2958.

Fig. 2.

The structural comparison of 5 PVL phages. Structures of ϕ108PVL, ϕPVL, ϕSLT, ϕSa2mw and ϕSa2958 are illustrated on the bases of the nucleotide sequences as follows: ϕ108PVL (DDBJ/EMBL/GenBank data bases under accession no. AB243556); ϕPVL (DDBJ/EMBL/GenBank accession no. AB009866); ϕSLT (DDBJ/EMBL/GeneBank accession no. NC_002661); ϕSa2mw (DDBJ/EMBL/GeneBank accession no. BA000033) and ϕSa2958 (DDBJ/EMBL/GenBank accession no. AP009363).

Genes having nucleotide identities of more than 90% are linked by blocks filled with each

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color: pink, genes conserved among 5 PVL phages; light purple, genes conserved among ϕSa2958, ϕSa2mw, and ϕSLT; blue, genes conserved between ϕPVL and ϕ108PVL. The positions of primers for M-PCR 1-7 are indicated by green arrowheads. Yellow and light green arrows indicate genes involved in phage morphological formation in ϕΣα2958, ϕSa2mw and ϕSLT; orange and dark green arrows indicate genes involved in phage morphological formation in ϕ108PVL and ϕPVL, respectively.

Fig. 3.

Representative results of 7 M-PCRs for identifying PVL phages. A 1-kb molecular weight marker (MWM) was run on both sides of the gel. Lanes; 1, PCR-1 using chromosomal DNA of 81/108; 2, PCR-2 using chromosomal DNA of JCSC2958; 3, PCR-3 using chromosomal DNA of 81/108; 4, PCR-4 using chromosomal DNA of JCSC2958; 5, PCR-5 using chromosomal DNA of 81/108; 6, PCR-5 using chromosomal DNA of ATCC49775; 7, PCR-6 using chromosomal DNA of JCSC2958; 8, PCR-7 using chromosomal DNA of MW2; and 9, PCR-8 using chromosomal DNA of RN4220 (ϕSLT).

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Table1. List of primers used in this study

Objectives of the primer construction	Primer's names	Nucleotide sequences(5'-3')	sizes (bp) of PCR product:	Genes or structures on which primers were designed	Locations of primers PVL phages
					[φSa2958]
For screening of fosmid Library	int-R2	CATTTTAATTGCCAGCATCTTA	558	*int*	1547..1525
	int-F2	ATGTTTTCGAGTTTTTGAGTTAG		*int*	989..1011
	PVL-F	ATGTCTGGACATGATCCAA	970	*lukS*-PV	45386..45404
	PVL-R	AACTATCTCTGCCATATGGT		*lukF*-PV	46356..46337
For long-range PCR to amplify entire strucure of φSa2958					
					[φSa2958]
From chromosome to integrase	phiMW2-DN1	GCAGAAAAAGATGCGATTGAA	(2.5 kb)	chromosome of JCSC2958	upstream to Φ2958PVL
	intR	AGGATATCGAAAAAGATGAATC		*int*	1655..1634
From integrase to DNA polymerase	intc	TTTGTAGTGTCTTTGTATCCG	9214	*int*	1376..1396
	MW1425-R	TTGTTGCCATTTTTCAAGATC		*pol*	10589..10569
From DNA polymerase to orf JP030	MW1425-F	CTAAAGTAGATAATGAGCCTT	8060	*pol*	10237..10257
	2958-1405R1	TCCCTTTTCTTGCTTCATTTC		JP030	18296..18276
From virulence-associated proteinE to portal protein	CF35	ACGAAGACGATTTTATCAAGG	6097	*por*	17509..17529
	2958-portalR1	CACTATATCTTCAGAGACATA		virE	23605..23585
From terminase large subunit to tail length tape me protein	CF47	AAAGTTATCTAATTCGATGGC	10480	*terL*	22990..23010
	CF67	GGGCTCTTGAATACATATCT		*mtp*	33469..33450
From tail length tape measure protein to orf JP052	2958-1392-F10	ATACTGAAAAGTGGTGGAATG	8741	*mtp*	32807..32827
	phi-M-TR1	GACTTCCTAAGTCGAAATAG		JP052	41547..41528
From orf JP052 to LukF-PV gene	phi-M-T1	TGGATTAACTAAATCTAGTCG	4877	JP052	41480..41500
	PVL-R	AACTATCTCTGCCATATGGT		*lukF*-PV	46356..46337
From orf JP058 to chromosome	LukS-RR	TGGTCAACTATATCGTGGTTTT	(2 kb)	JP058	44574..44595
	phiMW-UP	TCGCCACGTTTAGCAATTTTAT		chromosome of JCSC2958	downstream to φSa2958
For classifying phages					[φ108PVL]
PCR-1 (for identifying φ108PVL and φPVL)	portal-1F	ACACGTGATAAAACAGGAGAA	569	*por*	21069..21089
	portal-1R	TCTAAATTAGCATCCGTGATAC		*por*	21637..21616
	tail-1R	ATAATTGGGATAGCAACGCAA	489	*mtp*	31237..31257
	tail-1F	CTTGATTAGACTCAACCAAACT		*mtp*	31725..31704
					[φSa2958]
PCR-2 (for identifying φ2958PVL, φSLT and φSa2mw)	portal-2F	GATGGCTAGTTTGCCCTTGA	656	*por*	23005..23024
	portal-2R	CTGAGGGCAATTGAAAAACG		*por*	23660..23641
	tail-2F	CATAGCGCTAATGTCGCAAA	468	*mtp*	30040..30059
	tail-2R	AGCCTCCATTGTTTGTTTGG		*mtp*	30507..30488
					[φ108PVL]
PCR-3 (for identifying the gene lineage bewtween genes of φ108PVL and φPVL , and lukS-PV)	lukSR1	ACGAAGTAGCAATAGGAGTGA	10497	*lukS*-PV	42326..42306
	teil-ico-F	AGATTTAGAAGAGGAGGCACGA		*mtp*	31830..31851
					[φSa2958]
PCR-4 (for identifying the gene lineage bewtween genes of φSa2958, φSa2mw, and φSLT, and lukS-PV)	lukSR1	ACGAAGTAGCAATAGGAGTGA	9483	*lukS*-PV	44861..44841
	1	ATTGATTCAAACTGTTTCTTCTCAGGA		*mtp*	35379..35405
For identifying each PVL phage					[φ108PVL], [φPVL]
PCR-5 (for identifying φPVL and φ108PVL)	intF2	ATGTTTTCGAGTTTTTGAGTTAG	4340	*int*	393..415, 24310..24332
	108-aR	TCAAATCCGTAATCACTCATTCT		*ant*	4732..4710, -
	PVL-aR	TTCACTAACTAAACCTATCATTGT	1411	orf 30	- , 25720..25697
					[φSa2958]
PCR-6 (for identifying φSa2958)	int-F2	ATGTTTTCGAGTTTTTGAGTTAG	2238	*int*	989..1011
	2958-aR	TGGTAATCAACCATTCACTTATGA		JP004	3226..3203
					[φSa2mw]
PCR-7 (for identifying φSa2mw)	int-F2	ATGTTTTCGAGTTTTTGAGTTAG	4065	*int*	1574920..1574898
	MW2-aR	TAAGTTCCTGGTGTCATTCCTAAT		*cro*	1570856..1570879
					[φSLT]
PCR-8 (for identifying φSLT)	int-F2	ATGTTTTCGAGTTTTTGAGTTAG	8770	*int*	123..145
	SLT-aR	TCTTACCAAATGCAACACAACGAAT		*ssb*	8892..8868

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Table2.

Characterization of PVL-positive MRSA strains isolated in the years 1979-1985, 2002 and 2004.

Year of isolation (strains No.)	Phage type [a] (strains no.)	Coagulase isotype	SCC*mec* type (strains no.)	MLST Genotypes of chosen strains		
				no. of tested strains	ST	Allelic profile
1979-1985(53)	108PVL(36)	4	Type I (1)	1	30	2, 2, 2, 2, 6, 3, 2
			Type II (2)	1	30	2, 2, 2, 2, 6, 3, 2
			Type IV.1 (10)	3	30	2, 2, 2, 2, 6, 3, 2
				2	N[b]	2, 2, N, 2, 6, 3, 2
			Type IV.3 (23)	3	30	2, 2, 2, 2, 6, 3, 2
	2958PVL(14)	4	Type I (10)	2	30	2, 2, 2, 2, 6, 3, 2
			Type II (1)	1	30	2, 2, 2, 2, 6, 3, 2
			Type IV.3 (1)	1	30	2, 2, 2, 2, 6, 3, 2
			Type IV.n (1)	1	30	2, 2, 2, 2, 6, 3, 2
		2	Type II (1)	1	5	1, 4, 1, 4, 12, 1, 10
	non typeable (3)	4	Type IV.3 (2)	2	30	2, 2, 2, 2, 6, 3, 2
			Type IV.n (1)	1	30	2, 2, 2, 2, 6, 3, 2
2002(1)	2958PVL(1)	4	Type IV.3 (1)	1	30	2, 2, 2, 2, 6, 3, 2
2004(1)	2958PVL(1)	4	Type IV.3(1)	1	30	2, 2, 2, 2, 6, 3, 2

[a] Phage type judged by M-PCRs

[b] N, the sequence type (ST) of this strain could not be assigned, since the *glpF* gene could not be amplified by PCR. The allele numbers other than *glpF* were the same as those of ST30.

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Table 3.

Year of isolation (no. of strains)	identification of two kinds of phages (no. of strains)		identification of gene-lineage lukS-PV-taii gene (no. of strains)		identification of phage specific modules related to lysogeny and recombination	PVL-carrying phage type
	PCR-1(icosaheaderal shape head)	PCR-2 (elongated shape head)	PCR-3 (tail gene common to ϕ108PVL and ϕPVL)	PCR-4 (tail gene common to ϕSa2mw, ϕSLT, ϕSa2958)	PCR-5, -6, -7	
1979-1985(53)						
	+ (39)	+ (39)	+ (35)	ND	ϕ108PVL (33)	ϕ108PVL type (33)
					NT (2)	icosahedral head type (2)
			- (4)	ND	ϕ108PVL (4)	ϕ108PVL -like type (4)
	- (14)	+ (14)	ND	+ (1)	ϕSa 2958 (1)	ϕSa2958 type (1)
				- (13)	ϕSa 2958 (13)	ϕSa2958-like phage of unknown type (13)
2000s(12)	- (12)	+ (11)	ND	+ (10)	ϕSa 2958 (2)	ϕSa2958 type (2)
					NT (8)	elongated head type (8)
				- (1)	ϕSa 2958 (1)	ϕSa2958-like phage of unknown type (1)
		- (1)	ND	- (1)	ϕSa 2958 (1)	NT(1)

NT: non typeable

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Table 4. **Characterization of PVL-positive MRSA strains isolated in the years 1979-1985, 2002 and 2004.**

Year of isolation (number of strains)	Phage type[a] (number of strains)	Coagulase isotype	SCC*mec* type (number of strains)	MLST Genotypes of chosen strains		
				number of tested strains	ST	Allelic profile
1979-1985(53)	ϕ108PVL(33)	4	Type I (1)	1	30	2, 2, 2, 2, 6, 3, 2
			Type II (2)	1	30	2, 2, 2, 2, 6, 3, 2
			Type IV.1 (8)	3	30	2, 2, 2, 2, 6, 3, 2
				2	N[b]	2, 2, N, 2, 6, 3, 2
			Type IV.3 (21)	3	30	2, 2, 2, 2, 6, 3, 2
			Type IV.5(1)	1	30	2, 2, 2, 2, 6, 3, 2
	ϕ108PVL-like (4)	4	Type IV.1 (1)	1	30	2, 2, 2, 2, 6, 3, 2
			Type IV.3 (3)	1	30	2, 2, 2, 2, 6, 3, 2
	icosahedral head type (2)	4	Type IV.3 (1)	1	30	2, 2, 2, 2, 6, 3, 2
		4	type II.n (1)	1	30	2, 2, 2, 2, 6, 3, 2
	ϕSa2958 type (1)	2	Type II (1)	1	5	1, 4, 1, 4, 12, 1, 10
	Sa2958-like phage of unknown type (13)	4	Type I (10)	2	30	2, 2, 2, 2, 6, 3, 2
			Type II (1)	1	30	2, 2, 2, 2, 6, 3, 2
			Type IV.3 (1)	1	30	2, 2, 2, 2, 6, 3, 2
			Type IV.n (1)	1	30	2, 2, 2, 2, 6, 3, 2
2000s(12)	ϕSa2958 (2)	4	Type IV.3 (2)	2	30	2, 2, 2, 2, 6, 3, 2
	elongated head type (8)		Type IV.3 (1)	1	30	2, 2, 2, 2, 6, 3, 2
			NT (6)	1	30	2, 2, 2, 2, 6, 3, 2
	ϕSa2958-like phage of unknown type (1)	4	Type I (1)	1	765	2, 2, 2, 2, 6, 104, 2
	non typeable (1)	7	Type-V (1)	1	59	19, 23, 15, 2, 19, 20, 15

[a] Phage type judged with M-PCRs

[b] N, the sequence type (ST) of this strain could not be assigned, since the *glpF* gene could not be amplified by PCR. The allele numbers other than *glpF* were the same as t

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Table 4. **Identification of PVL-phages induced by mitomycin C treatment**

Strains	Coagulase isotype	MLST	SCC*mec* type	Phage type[a]	indicator strain	No. of phages induced by mitmycin C p. f. u./ml	No. of tested plaques	No. of PVL-positive plaques (%)
MW2	7	1	IV.1	ϕSa2mw(S)	RN4220	2.34×10^{6}	234	234 (100 %)
					1039	3.86×10^{6}	386	386 (100 %)
JCSC2958	2	5	II	ϕSa2958 (S)	RN4220	2.26×10^{9}	226	226 (100%)
					1039	1.05×10^{10}	316	316 (100 %)
RN4220/ϕSLT	3	8	-	ϕSLT(S)	RN4220	1.11×10^{6}	111	111 (100 %)
					1039	2.23×10^{5}	223	223 (100 %)
81/108	4	30	IV.3	ϕ108PVL(S)	RN4220	4.2×10^{2}	42	0
					1039	1.82×10^{5}	182	0
JCSC2973	4	30	I	ϕSa2958-like(P)	RN4220	2.32×10^{5}	232	0
					1039	4.08×10^{6}	408	2 (0.49%)
M11	4	30	IV.3	ϕSa2958-like(P)	RN4220	2.05×10^{8}	205	205 (100%)
					1039	2.33×10^{8}	233	233 (100%)
JCSC4465	4	30	IV.n	ϕSa2958-like(P)	RN4220	1.34×10^{8}	134	0
					1039	1.48×10^{8}	148	4 (2.7%)
JCSC4625	4	30	IV.3	ϕSa2958 type(P)	RN4220	2.61×10^{5}	261	261 (100%)
					1039	3.97×10^{6}	397	397 (100%)
JCSC6055	4	30	IV.3	ϕSa2958 type(P)	RN4220	no plaque	NT	NT
					1039	no plaque	NT	NT

[a] Phage types assinged with PCR are indicated by (P), and those judged by sequences of phage genomes are indicated by (S).

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Fig. 1

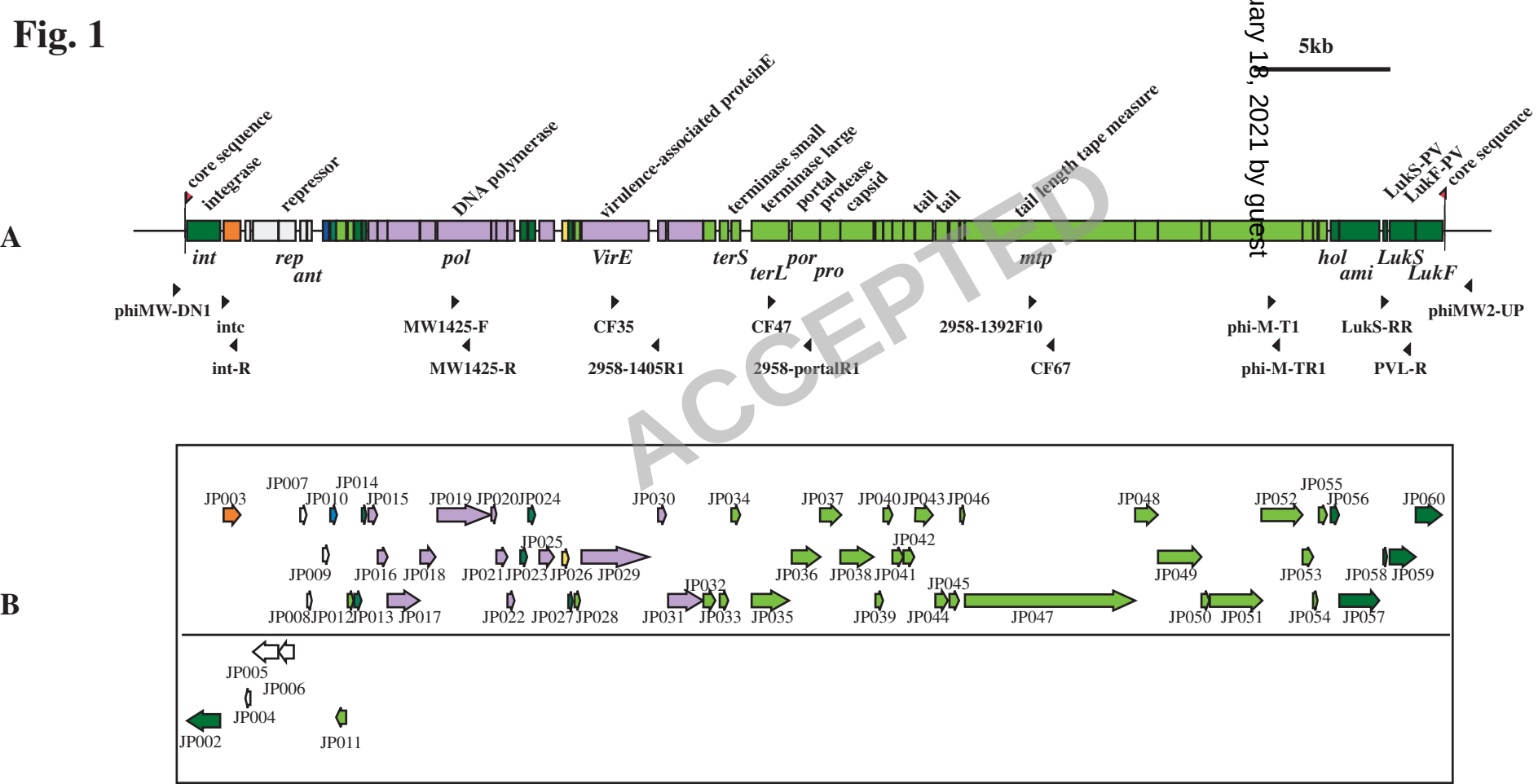

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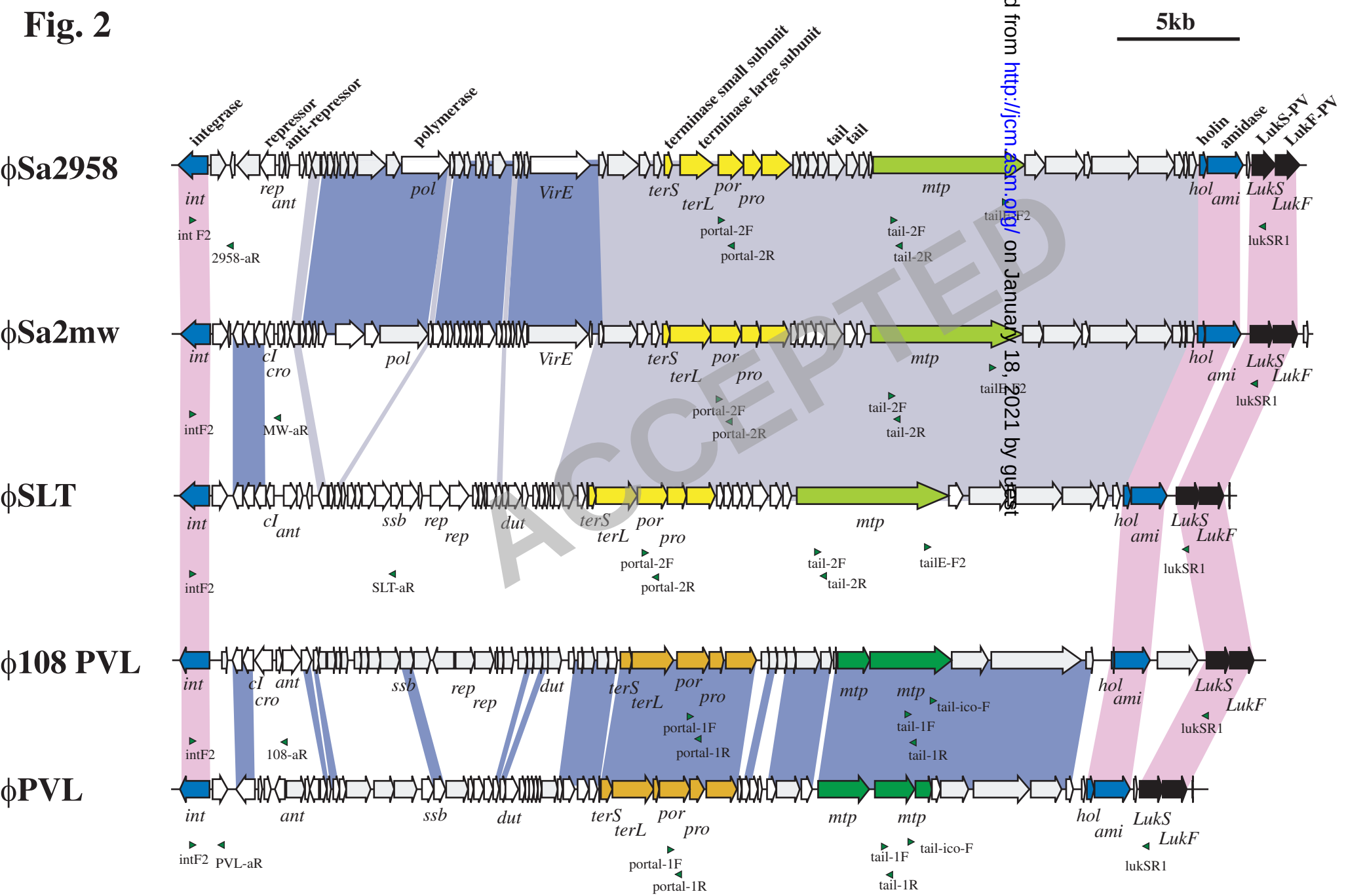
Fig. 2



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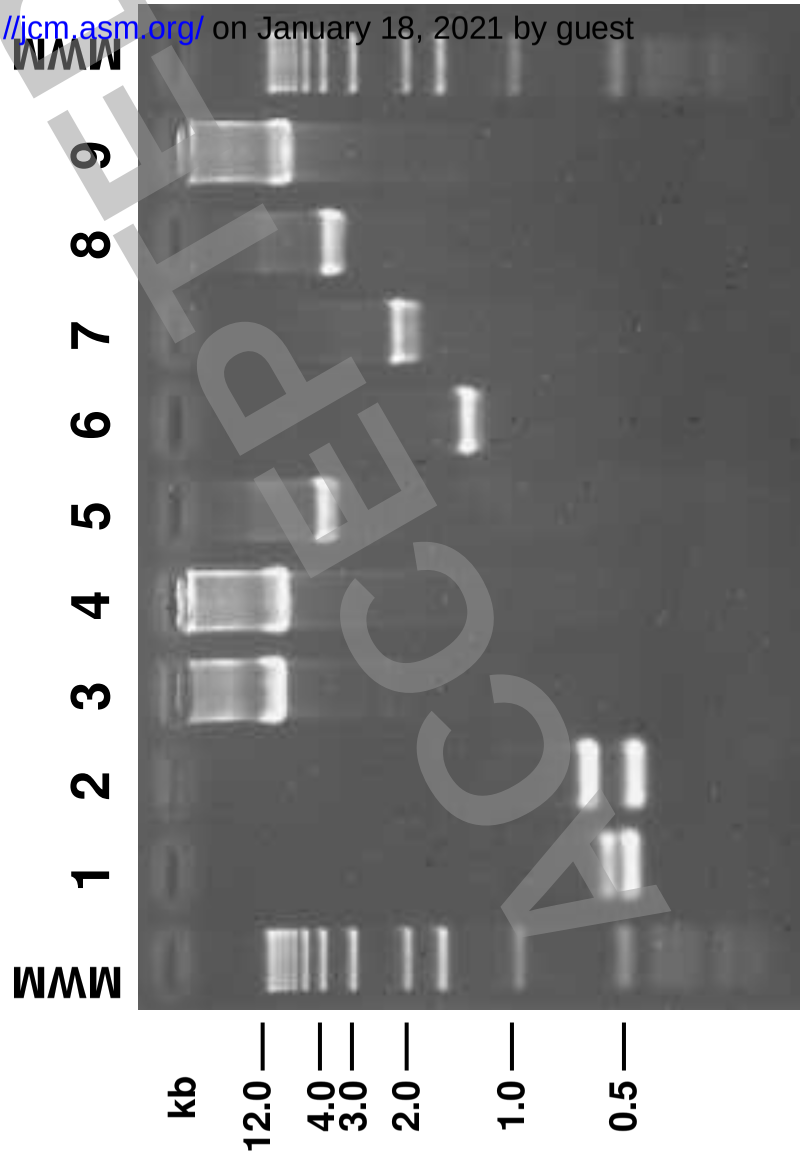

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