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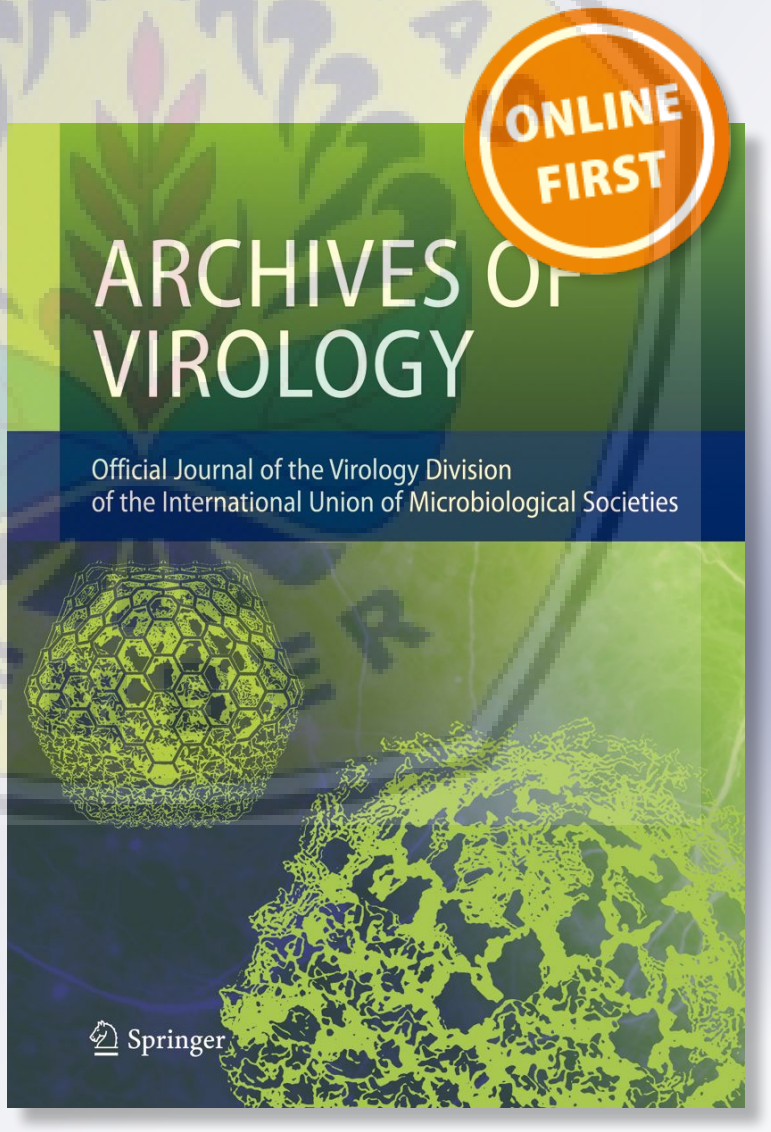

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BRIEF REPORT

Host range and molecular characterization of a lytic *Pradovirus*-like Ralstonia phage RsoP1IDN isolated from Indonesia

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Abstract

A lytic *Ralstonia solanacearum*-infecting phage designated Ralstonia phage RsoP1IDN was isolated from soil in Indonesia. The phage has a linear double-stranded DNA genome of 41,135 bp with 413-bp terminal repeats, and contains 41 annotated open reading frames. The phage is most closely related to Ralstonia phage RSB1, but different from RSB1 mainly in containing a putative HNH homing endonuclease and having a narrower host range. Our phylogenetic and genomic analyses revealed that both phages RsoP1IDN and RSB1 belong to the genus *Pradovirus* or a new genus, and not *Phikmvvirus* as previously reported for phage RSB1. RsoP1IDN is the first sequenced and characterized *R. solanacearum*-infecting phage isolated from Indonesia in the proposed species *Ralstonia virus RsoP1IDN*.

Keywords: *Ralstonia solanacearum* · *Podoviridae* · *Pradovirus* · Bacterial wilt · Phage therapy

Ralstonia solanaceraum is a Gram-negative, soil-borne and destructive phytobacterium attacking more than 450 plant species from over 50 botanical families [1]. Traditionally, *R. solanacearum* is classified into five races and five biovars. Molecular classification, however, has grouped strains of *R. solanacearum* into four phylotypes and 53 sequevars [2, 3]. *R. solanacearum* is considered a species complex [4], and effort has been made to divide it into three different *Ralstonia* species [5]. Such a reclassification of the *R. solanacearum* species complex was supported by a recent study using genomic, proteomic and functional phenotypic approaches [6]. Control of *R. solanacearum* by cultural practices has not been successful due to the pathogen's wide host range, broad distribution, great genetic variability and ability to survive in soil. Chemical control of *R. solanacearum* is not practical, high in cost and has negative effects on the environment. Recently, efforts have been made to explore the possibility of using phage therapy for control of *R. solanacearum* [7–9]. So far, many bacteriophages specifically infecting *R. solanacearum* have been isolated and reported including phages of the family *Inoviridae, Myoviridae*, *Siphoviridae*, and *Podoviridae* such as phages RSB1 [10], RSB2, RSB3, RSJ2, RSJ5 [8], RS-PI-1, RS-PII-1 [11], RsoP1EGY [12], and unpublished phage phiTL-1 (GenBank accession no. KP343639.1). The potential of using some of the phages to control *R. solanacearum* has been studied [7, 8, 13, 14].

In this study, a *R. solanacearum*-infecting phage was isolated from the soil of an eggplant field in Jember, East Java, Indonesia using the method of Bhunchoth et al. [8]. A single plaque was picked, serially diluted in SM buffer, and subjected to plaque assay [15, 16] using *R. solanacearum* strain DT3 as a host. The procedure was repeated two more times for a triple phage purification process to obtain a pure phage isolate. The host specificity of the phage was tested

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Table 1 Susceptibility of *Ralstonia solanacearum* strains to phage RsoP1IDN

R. solanacearum strains	Biovar/ phylotype-sequevar	Origin	Susceptibility to phage RsoP1IDN
AW1	1/IIA-7	USA	R
K60	1/IIA-7	USA	S
P550	1/IIA-7	USA	R
Rs5	1/IIA/7	USA	R
RUN036	1/IIA-36	Martinique	R
RUN302	1/ IIB-4	Brazil	S
RUN133	2T/II-29	Cameroon	R
UW224	2/IIB-1	Kenya	R
UW257	2/IIB-1	Costa Rica	R
UW344	2/IIB-1	Brazil	R
UW550	2/IIB-1	Netherland	R
UW552	2/IIB-1	Guatemala	R
DT3	3/I	Indonesia	S
HB512	3/I	China	R
Pss530	3/I	Taiwan	R
UW152	3	Australia	R

R: resistant. S: susceptible

against sixteen *R. solanacearum* strains (Table 1), either through plaque assay or spot test on 0.45% of soft agar [15, 17]. The phage has a narrow host range infecting only three of the 16 tested *R. solanacearum* strains (Table 1) as large clear plaques (i. e an average single plaque size of approximately 9.15 mm ± 1.63 (n=20) in diameter on the lawn of *R. solanacearum* strain DT3). The phage was purified by ultracentrifugation through a 30% sucrose cushion as described by Ahmad et al. [15] and treated with an equal volume of chloroform before examination under a Hitachi 7700 transmission electron microscope. The phage virions have an icosahedral head with a diameter of 62 ± 4 nm (n=15) and a short non-contractile tail with a length of 17 ± 2 nm (n=15), a typical morphology for members of the *Podoviridae* (Fig. 1A). Similar to the phage identifier naming approach we used before [12], the phage was designated Ralstonia phage RsoP1IDN to reflect its bacterial host species, morphology and origin, since it is the first *R. solanacearum*-infecting bacteriophage belonging to the family *Podoviridae* that was isolated from Indonesia.

The genome of Ralstonia phage RsoP1IDN was extracted from purified phage particles described above using the Phage DNA Isolation kit (Norgen Biotek Corp, Canada), and subjected to enzymatic digestions using standard molecular biology methods. The phage genome was completely degraded by DNase I, but not by RNase A, S1 nuclease, or Exonuclease I (ThermoFisher, USA) (Fig. S1). The genome was also digested into multiple bands by endonuclease *Xho*I (BioLabs, USA) (Fig. S1). These results indicate that phage RsoP1IDN has a double stranded DNA genome. The phage DNA was sequenced and assembled commercially by SeqMatic (Fremont, California). The 40,722-bp circular map of the phage was generated using DNASTAR (DNASTAR Inc, USA) and is presented in Fig. S2. Since podoviruses are known to contain terminal repeats [10, 18, 19], efforts were made to determine how long the terminal repeats were, in order to obtain the linear genome size of phage RsoP1IDN. This was done by first predicting where the repeat region was based on the genomic organization/ arrangement of phage RsoP1IDN as compared to closely related phages and the partially repeated-sequence at both ends of the assembled phage genome. Primers were then designed upstream and downstream towards the predicted repeat region (DT3tRep-1KB-F: 5'-CCT CGT AAA TCG ACG CCA GA-3'; DT3tRep-1KB-R: 5'-GGC TCG ATC TTG ATG GCG TA-3'), followed by sequencing using phage genomic DNA as a template, which determined that the terminal repeat on both ends was 413-bp. The complete linear nucleotide sequence of phage RsoP1IDN consists of 41,135-bp including the 413-bp terminal repeats at both ends, and has a G+C content of 62.72% (GenBank accession no. MG652450). When the complete genome sequence of phage RsoP1IDN was used as a query to search GenBank by BLASTn, the phage was found to be most closely related to the Ralstonia phage RSB1 [10] with 84% nucleotide identity over 82% coverage.

Potential open reading frames (ORFs) larger than 80 amino acids (aa) were identified in the genome of phage RsoP1IDN using the phage prediction software PHASTER [20] and further analyzed using DNASTAR (DNASTAR Inc, USA). Homology searches for each identified ORF were performed using BLASTp [21] against NCBI's protein databases. The e-value threshold of 10^{-4} or less was used for two proteins to be considered a match. Forty-one ORFs were predicted (Table S1, Fig 2, and Fig. S2) by PHASTER with start codon of ATG (35/41), GTG (5/41) or TTG (1/41) and stop codon of TAG (20/41), TAA (18/41), or TGA (3/41). They are all transcribed from the same strand (Fig. 2 and Fig. S2). Their positions, best homologs and predicted functions are summarized in Table S1. Among them, 31 had sequence identity (59-97%) with Ralstonia phage RSB1, and six (42-72%) with Ralstonia phages RSJ5 and RSK1, Sinorhizobium phage phiM12, Bacillus phage BCP8-2, as well as genome sequences of *R. solanacearum* and *Paraburkholderia fungorum* (Table S1). Four had no similarity with any proteins in the databases (Table S1).

Similar to other podoviruses, three functional gene clusters [10, 18, 19] were identified in the genome of phage RsoP1IDN: cluster I for early genes including DNA helicase (ORF9); cluster II for metabolism genes including two DNA polymerases (ORFs 13 and 15), DNA primase (ORF8), DNA endonuclease VII (ORF16); and cluster III

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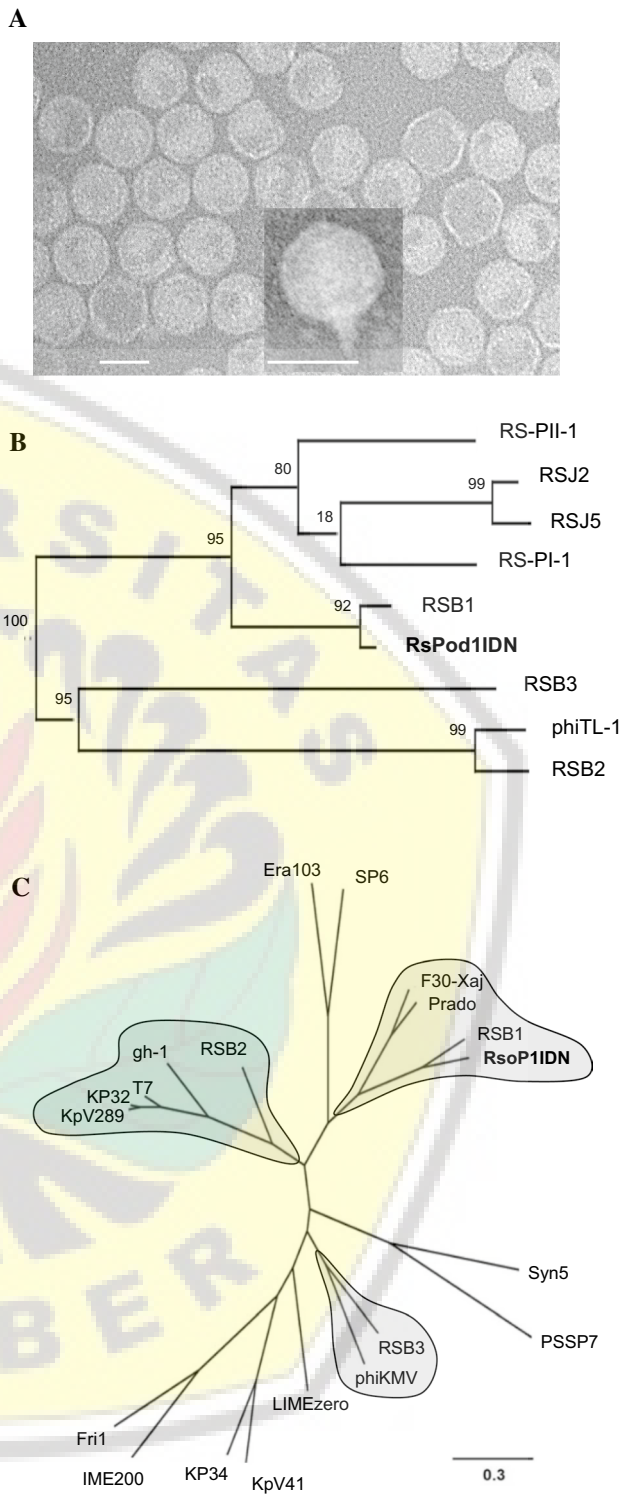

Fig. 1 Morphology of the Ralstonia phage RsoP1IDN and its phylogenetic relationships to other phages. (A) Electron micrograph of phage RsoP1IDN's virions. The scale represents 50 nm. (B) Phylogenetic relationship between Ralstonia phage RsoP1IDN and other *Ralstonia* phages of the family *Podoviridae* based on deduced amino acid (aa) sequences of RNA polymerase. Vertical distances are arbitrary, but the horizontal branches are proportional to genetic distance. Bootstrap values (1000 replicates) are represented at the nodes of the branches. (C) Phylogenetic relationships among Ralstonia phage RsoP1IDN, the three RSB *Ralstonia* phages and 16 non-*Ralstonia* phages from eight genera of the subfamily *Autographivirinae* of the family *Podoviridae*, based on deduced aa sequences of RNA polymerase. Phylogenetic trees were generated using the free online service Phylogeny.fr [22]. The scale represents genetic distance

for structural and assembly genes including six genes for head and tail morphogenesis (ORFs 26-30 and 34), putative transglycosylase (ORF33), and large terminase subunit protein (ORF38) (Table S1). Gene functions for host-cell lysis such as transglycosylase (ORF33), holin T7 family (ORF36), and putative lysozyme (ORF40), but not functions for phage integration, were identified in phage RsoP1IDN (Table S1), suggesting that like most of podoviruses, phage RsoP1IDN is a lytic phage that lyses bacterial hosts without a lysogenic stage.

In addition to ORFs, potential promoters and transcriptional terminators were predicted in the genome of phage RsoP1IDN using online programs Neural Network Promoter Prediction [22] with a minimum promoter score of 0.9 and ARNold [23], respectively. Six putative prokaryotic promoter sequences (p1 – p6) similar to *E. coli* σ^{70}-like promoter sequence were predicted in the 2,569-bp non-coding region downstream of ORF1 (Figs. S2 and S3). Such tandem *E. coli* σ^{70}-like promoter sequences were also found in Ralstonia phage RSB1 [10]. In addition, two other putative *E. coli* σ^{70}-like promoter sequences (p7 and p8) were predicted upstream of ORF13 (DNA polymerase) and ORF28 (major coat protein), respectively (Figs. S2 and S3). Four putative rho-independent transcriptional terminators were identified after ORFs 3 (hypothetical protein), 22 (RNA polymerase), 28 (major capsid-like protein), and 38 (terminase) (Figs. S2 and S3). The fact that ORF28 has its own predicted promoter and transcriptional terminator strongly suggests that the deduced major coat protein of phage RsoP1IDN is encoded or expressed independently.

Phylogenetic relationships between phage RsoP1IDN and eight other *Ralstonia* phages of the family *Podoviridae*, including RSB1, RSB2, RSB3, RSJ2, RSJ5, phiITL-1, RS-PI-1, and RS-PII-1, were estimated using the free "One Click" web service Phylogeny.fr [24]. The references, if any, and GenBank accession numbers for the aa sequences of RNA polymerase and the complete genome sequences of the phages used in this study were presented in Table S2. The phylogenetic tree derived from the deduced aa sequences of RNA polymerase showed that phage RsoP1IDN is more closely related to the Ralstonia phage RSB1 than to other *Ralstonia* phages (Fig. 1B). A similar tree was obtained when the deduced aa sequences of DNA polymerase, major coat protein, tail fiber protein, terminase, or lysozyme [25, 26] were used to do the analysis (data not shown), further support the close phylogenetic relationship between phages

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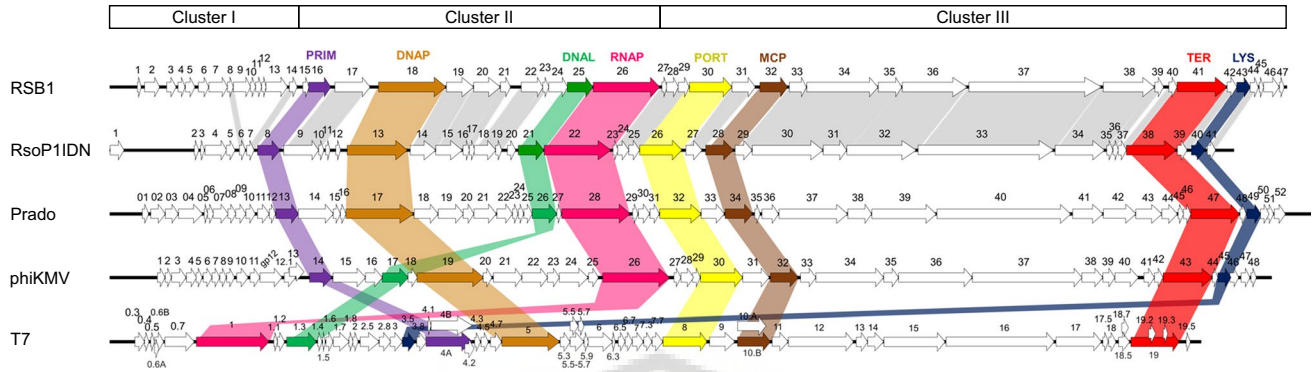

Fig. 2 Comparison of genomic organization between phages RsoP-1IDN and RSB1, as well as between phage RsoP1IDN and phages from the genera *Pradovirus* (Xylella phage Prado), *Phikmvvirus* (Pseudomonas phage phiKMV), and *T7virus* (Enterobacteria phage T7). Arrows represent size and direction of transcription of ORFs. The three functional gene clusters are indicated at the top of the phage genomes. The location of conserved phage ORFs such as DNA primase (PRIM, purple), DNA polymerase (DNAP, orange), DNA ligase (DNAL, green), RNA polymerase (RNAP, pink), Phage portal protein (PORT, yellow), major coat protein (MCP, brown), large terminase subunit (TER, red), and lysozyme (LYS, blue) are compared. ORFs with the same predicted functions are connected by light grey shading between phages RsoP1IDN and RSB1

RsoP1IDN and RSB1. Phage RsoP1IDN, however, is different from phage RSB1 in host range, since the formal has a narrow (Table 1) but the latter broad host range [10]. In addition, phage RsoP1IDN is different from phage RSB1 in genomic organization and gene structure in the following ways. (i) The order of the early genes in the Cluster I region of phage RsoP1IDN begins with ORF1, then the 2,569-bp non-coding region, followed by ORFs 2 to 5, while the order of the early genes of phage RSB1 begins with a 996-bp non-coding region, followed by ORFs 1 to 7 before the homologous ORFs between the two phages (Fig. 2). (ii) Phage RsoP1IDN has fewer ORFs in Clusters I and II than phage RSB1 (Fig. 2). (iii) The putative HNH homing endonuclease gene, found in some podoviruses, was present in phage RsoP1IDN (*orf12*), but not in phage RSB1 (Fig. 2). This ORF has no homologs in other *Ralstonia* phages, but instead shares sequence similarity with Bacillus phage BCP8-2 (Table S1). (iv) ORF36 of phage RsoP1IDN has similarity with the holin T7 family, but no holin genes were annotated in phage RSB1. Similar to ORF12, ORF36 shared no sequence homology with any *Ralstonia* phages or prophages in *R. solanacearum*. Instead, it showed sequence identity with *Paraburkholderia fungorum* (Table S1).

Currently, 8 genera are listed under the subfamily *Autographivirinae* of the family *Podoviridae* (https://talk.ictvonline.org/taxonomy, last accessed on May 15, 2018) (Table S2). Previously, *Ralstonia* RSB-like phages such as RSB1, RSB2 and RSB3 were grouped into two types: RSB2-type which is a T7-like phage of the genus *T7virus* and RSB1- and RSB3-types which are ϕKMV-like phages of the genus *Phikmvvirus* [27]. When the aa sequences of RNA polymerase were used to determine the phylogenetic relationships among phage RsoP1IDN, the three RSB phages [10, 27] and 16 other representative non-*Ralstonia* phages from each of the eight genera of the subfamily *Autographivirinae*, phage RsoP1IDN was again found in the same clade with phage RSB1 (Fig. 1C). Unexpectedly, however, both phages RsoP1IDN and RSB1 were grouped together with phages from the genus *Pradovirus* including Xylella phage Prado [28] and Xanthomonas phage f30-Xaj [29], not with phages from the genus *Phikmvvirus* like phage RSB3 and the Pseudomonas phage phiKMV [19] (Fig. 1C). This result differs from previous reports that phage RSB1 is T7-like [10] or ϕKMV-like [27]. Phage RSB2 was grouped with phages of the genus *T7virus* such as Enterobacteria phage T7, Pseudomonas phage gh-1, and Klebsiella phages KP32 and KpV289 as expected (Fig. 1C). To confirm the results, we compared the genome organization of phages RsoP1IDN and RSB1 against three other phages representing the genera *Pradovirus* (Xylella phage Prado) [28], *T7virus* (Enterobacteria phage T7) [18] and *Phikmvvirus* (Pseudomonas phage phiKMV) [19] (Fig. 2). Phages RsoP1IDN and RSB1 are similar in genomic organization and the arrangement of ORFs, with greater resemblance to the Xylella phage Prado of the genus *Pradovirus* than to the Pseudomonas phage phiKMV of the genus *Phikmvvirus* and least of all to the Enterobacteria phage T7 of the genus *T7virus* (Fig. 2). This is especially true for the position or arrangement of ORFs encoding for DNA primase, DNA polymerase, DNA ligase, RNA polymerase, phage portal protein, major capsid protein, terminase and lysozyme (Fig. 2). It is noteworthy that the position of the putative RNA polymerase in phage RsoP-1IDN (ORF22) is located right after DNA ligase (ORF21), as in phage RSB1 and Xylella phage Prado, while separated by 7 upstream ORFs from DNA ligase in the Pseudomonas phage phiKMV, and 2 ORFs upstream of the DNA ligase in

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the Enterobacteria phage T7 (Fig. 2). Based on our phylogenetic and genomic organization results, phages RsoP1IDN and RSB1 either belong to the genus *Pradovirus*, or form a new, not yet classified genus. These two phages, however, are different at the species level, since they share 84% nucleotide sequence identity, more than the 5% difference in DNA sequence conventionally used as a criterion for demarcation of species. In addition, the two phages are different in host range, genomic organization and gene structure as mentioned above. As a result, the Ralstonia phage RsoP1IDN should be considered a new species with a proposed species name of *Ralstonia virus RsoP1IDN* in the family of *Podoviridae*.

In conclusion, the lytic Ralstonia phage RsoP1IDN was isolated from Indonesia. The phage has a double stranded DNA genome of 41,135 bp in size and is most closely related to the Ralstonia phage RSB1. Our phylogenetic and genome organization studies revealed that phages RsoP1IDN and RSB1 belong to the genus *Pradovirus* or a new genus, and not *Phikmvvirus* as previously reported for phage RSB1 [27]. Phage RsoP1IDN has potential as a biocontrol agent for *R. solanacearum* due to its lytic nature. Its effectiveness, however, may be limited due to the narrow host range of the phage. Phage RsoP1IDN also has potential to serve as a source for antimicrobial proteins. RsoP1IDN is the first sequenced and characterized *R. solanacearum* phage isolated from Indonesia in the proposed species *Ralstonia virus RsoP1IDN*.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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