Existence of a colonizing Staphylococcus aureus strain isolated in diabetic foot ulcers

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Running title: Diabetic foot and Staphylococcus aureus
Abstract

*Staphylococcus aureus* is an opportunistic bacterium capable of causing a wide range of severe diseases when it gains access to underlying tissues. Paradoxically, *S. aureus* is a common inhabitant of the skin microflora and colonizes the nares and other human mucosa. The purpose of this study was to determine genetic basis for the differences in pathogenic versus colonizing potential of *S. aureus* isolated from diabetic foot ulcers (DFU). By performing optical map comparisons of a collection of *S. aureus* strains isolated from DFU, we brought to light a prophage present in non-infecting bacteria. The phage, namely ROSA-like, was localized in a hotspot region ΦNM2 near the locus *isd*, the iron surface determinant system. The integrated phage reduces significantly the virulence of the strain and increases the biofilm formation. DFU seems to be a specific niche of this colonizing strain. The ROSA-like phage represents the first description of a mobile element present mainly in *S. aureus* isolated from DFU, which modulates the relationship of the bacteria with its human host. This phage appears to attenuate bacterial virulence and to promote colonization.

**Key word**: Diabetic foot ulcer, colonizing, infection, optical maps, phage, *Staphylococcus aureus*, virulence.
*Staphylococcus aureus* is by far the most common and virulent pathogen in diabetic foot infection (1,2). However this causative pathogen is a common inhabitant of the skin microflora and colonizes the nares and other human mucosa. It may be considered as an opportunistic colonizing organism. Recently, we demonstrated the coexistence of two populations of *S. aureus* strains isolated from diabetic foot ulcer (DFU): strains isolated from uninfected ulcers with a low virulence potential as opposed to strains isolated from infected ulcers with a high virulence potential (3,4). Moreover the strains belonged to two clonal complexes (CC) CC8/CC5 that appeared to be linked to uninfected ulcers, enabling us to distinguish uninfected from infected wounds (5). In this study we describe for the first time an insertion of a phage in the CC8 lineage of methicillin-sensitive *S. aureus* (MSSA) which is associated with the colonizing *S. aureus* strains, and we report the impact of this phage on biofilm formation and bacterial virulence.

### RESEARCH DESIGN AND METHODS

**Bacterial strains and plasmids**

All bacterial strains used in this study are listed in Table 1. Bacteria were grown at 37°C in Luria Bertani broth (LB) or Brain Heart Infusion broth (BHI).

**Caenorhabditis elegans** and zebrafish killing models

Fer-15 worms were maintained and infected as previously described (6). All experiments were conducted in triplicate and repeated at least five times for each strain. *S. aureus* virulence was assessed using the nematode survival curve and calculating the LT50 and LT100 (median lethal time 50% and 100%, respectively).

The presence of *S. aureus* in the *C. elegans* digestive tract was determined at 72h as described by Garsin *et al.* (7). Three replicates were performed for each strain. Infection of zebrafish embryos was carried out as previously described (8). More information regarding the two models can be found in Supplementary Data.
Optical maps

Twenty-two strains from our panel of colonizing and infecting *S. aureus* strains isolated from DFU (5) were chosen for optical mapping. Optical maps were kindly provided by OpGen (Gaithersburg, MD USA), prepared on the ArgusTM Optical Mapping System as described previously (9) and analyzed with the support of Phylogen (Bernis, France). The optical maps of the studied strains were then compared with the *in silico* restriction maps of 19 of sequenced *S. aureus* isolates whose sequence genomes were available in GenBank and transformed by using the MapSolver v.2.1.1 software (OpGen SA).

Sequencing of the NSA1385 strain and the 44kb insertion

Genomic DNA of *S. aureus* NSA1385 was sequenced using a 454 Life Science-Roche platform by Lifesequencing S.L., Valencia (Spain). The combination of scaffolds and contigs resulted in an estimated genome size of 3.2 Mb.

PCR for the detection of phage insertion/deletion and sequencing

The PCR protocol was presented on supplementary data. After purification, PCR products were sequenced using a Perkin-Elmer ABI377 sequencer and compared with sequences in Genbank by BLAST (http://www.ncbi.nlm.nih.gov/blast).

Biofilm formation

To evaluate the biofilm formation, we used the Biofilm ring Test® (BioFilm Control®, Saint Beauzire, France) following the manufacturer recommendations (10). Three experiments with three repeats each (3 wells per slide) were performed per strain and incubation time.

Evaluation of spontaneous phage excision
To detect and evaluate spontaneous excision of the ROSA-like phage from the hotspot region $\Phi$NM2, we used different procedures already described: the TMS medium with or without FeCl$_3$ (50 $\mu$M) to create iron-repleted and iron-restricted growth conditions, mitomycin C and UV treatment (11-13). We evaluated the occurrence frequency of NSA1385 mutants that lost the phage by counting the number of NSA1385 without phage CFU and compared to the NSA1385 CFU number. Phage excision was confirmed using a PCR assay and genome sequencing.

Statistical analysis
The Mann-Whitney test was used to compare the in vivo bacterial growth of the different strains. To compare overall survival curves in nematode and zebrafish killing assays, a Cox regression was used. For pairwise comparison of two survival curves in nematode and zebrafish killing assays, we used a log rank test. Statistical analysis was performed using S-Plus 2000 software package (Insightful Corporation, Seattle, WA, USA) and results were considered significant at p<0.05.

RESULTS
Non-infecting strains are less virulent than infecting strains
We used two infection models to confirm our previous observations that clinical S. aureus strains isolated from Grade 1 DFU are less virulent than strains isolated from Grade 2-4 DFU (4). We analyzed the behaviour of five clinical strains: the two uninfecting strains (NSA1322, NSA1385, from Grade 1 DFU), the two infecting strains (NSA739, NSA18026 from Grade 2-4 DFU) and the reference strain Newman. In the C. elegans model, the five studied strains killed the nematodes more rapidly than the avirulent E. coli OP50 strain used as nutrient for the nematodes ($p<0.001$) (Table 1). The LT50 were similar for the two colonizing strains and the strain Newman but significantly longer ($p<0.001$) than the LT50 of the infecting strains (4.0-4.6 days ±0.3 vs 1.6-1.7 ±0.2,
respectively; Table 1). The differences in virulence were not due to differences in the survival and proliferation of strains within the nematode intestine, since the intestine colonization by the different strains was not significantly different (Table 1).

Based on the results obtained with *C. elegans*, we investigated whether the two uninfecting strains (NSA1322, NSA1385) also exhibit lower virulence in zebrafish embryos. We compared mortality rates over a 92-hour period. The two colonizing strains caused less embryo deaths than the two infecting strains (*p*<0.001) (Figure 1A-B).

**The non-virulent colonizing *S. aureus* strains carry a genetic island**

To investigate the difference between colonizing and infecting strains isolated from DFU (4), we used optical mapping to analyze their genome organization (Figure S1). The 5 colonizing strains were clonal (>99% similarity) and clustered closely to two reference strains (Newman and NCTC8325) with approximately 98% similarity. The majority of the infecting strains belonged to different clonal groups with less than 90% similarity to the colonizing strains. Interestingly, five infecting strains (NSA739, NSA6759, NSA11260, NSA18026 and NSA56348) exhibited 96.5% similarity with the colonizing strains. All these strains belonged to the CC8-MSSA clonal complex.

The major difference between the colonizing and the infecting CC8-MSSA strains was the presence of large insertion located exclusively in all of the colonizing strains (CC8- and CC5-MSSA). The insertion was located at the previously described ΦNM2 integration hotspot (Figure S2), a known hotspot for genetic insertions, with phage insertions identified in the published genomes of the two reference strains (Newman and NCTC 8325) (14).

**The 44 kb-genetic island corresponds to a ROSA-like phage**
To characterize the genetic insertion present in the colonizing strains, a draft genome sequence of NSA1385 was determined to approximately 29X coverage. The integration site is in the intergenic region between rpmF (encoding the 50S ribosomal protein L32) and isdB (encoding the staphylococcal haemoglobin receptor required for heme-iron uptake). The insertion has a high G+C content (35.4%, compared with 32% for the chromosome in total), and appears to be an integrated bacteriophage. This phage has a genome of 44,031 bp with 73 ORFs, and is allocated within unclassified dsDNA phages group. The comparison of the phage sequence with sequences in Genbank highlighted that the phage integrates at the same sequence as the phage ROSA, a previously described phage (15) with no known function. The sequences were comparable but we noted an inversion of a part of the phage sequence (Figure S2). We named this genetic island, ROSA-like. More information can be found in Supplementary Data.

The genetic island is associated with colonization of chronic wounds in France

To evaluate whether the ROSA-like phage insertion is a common feature from uninfecting strains, we tested a collection of S. aureus strains isolated in different clinical situations in France (Table 2). The insertion was identified in 40/392 strains (10.2%) of our collection with 39/75 (52%) from colonizing DFU and 1/131 (0.8%) from nose. The ROSA-like phage was almost exclusively found in Grade 1 ulcers (39/44 uninfected wounds, 88.6%) and all the strains harbouring the insertion belonged to the CC8/CC5-MSSA lineages.

The genetic island promotes biofilm formation

To determine the impact of the genetic insertion, we studied biofilm formation using the BioFilm Ring Test®. In BHI medium, we observed that the colonizing NSA1385 strain formed biofilms more rapidly than the infecting NSA739 strain (150 min vs 180 min) (Figure 2). These results confirmed the colonizing role of the investigated strains.

The ROSA-like phage is very stable in lysogens
An important issue in clinical practice is to know if the Rosa-like phage is stable or not. Using different DNA damaging stimuli, we observed that the frequency of excision was $<5 \times 10^{-9}$, suggesting a strong selective pressure for its maintenance. One strain with a confirmed loss of the ROSA-like phage (detected by PCR, optical map and DNA sequencing) was selected for further experiments and named NSA1385(P-).

**The excision of the ROSA-like phage restores biofilm formation and bacterial virulence**

To definitively understand the impact of the phage insertion in the colonizing strain, we evaluated the biofilm formation and the virulence of NSA1385(P-). We observed the restoration of biofilm formation (Figure 2) and bacterial virulence using the two *in vivo* models (Table 1, Figure 1B). All these data suggest that the ROSA-like phage clearly influences the virulence of the colonizing *S. aureus* strain. The absence of the phage restores the bacterial virulence. However the low level of excision indicates that the ROSA-like phage is very stable and suggests that this colonizing strain does not require antibiotic treatment.

**DISCUSSION**

*S. aureus* is one of the most frequent pathogens isolated from community-acquired and nosocomial infections and the most prevalent in DFU (2). Even though *S. aureus* strains can colonize different human mucosa and may be considered as a commensal organism, these bacteria are clearly pathogens. However, we recently described the existence of a colonizing *S. aureus* strain isolated from DFU (4). Here, a comparative genomic strategy using a collection of clinical strains detected a genetic determinant (a ROSA-like phage) associated with the attenuation of the clonal group. To confirm that this element was responsible for the colonizing behavior, we assessed the distribution of the ROSA-like phage in disease-causing and asymptotically carried *S. aureus* in a national, nonbiased population taken from national epidemiological studies. The results demonstrated, for the first time, that the prophage was associated with the ability of the bacteria to colonize chronic ulcers and was responsible for the non-invasive character.
The carriage of virulence determinants by phage is not an uncommon situation in bacterial pathogens (16-20). In *S. aureus*, many of the phages encode and disseminate potent staphylococcal virulence factors (e.g. Panton-Valentine Leukocidin) or resistance determinants (SCCmec cassette) (21). However, more recently, authors have shown that some phages could affect bacterial virulence by preventing the production of toxins (22). In our study, we demonstrated that the phage insertion blocks the virulence potential. Moreover, in the colonizing *S. aureus* strain, the difficulties to induce phage excision (using DNA-damaging agents), the low phage excision frequency and the different rearrangements noted (compared to the published genome sequence of phage ROSA) demonstrated a great stability of this insertion suggesting a low ability to spread or transfer to other staphylococci.

The association with a high potential of biofilm formation and the avirulence of the *S. aureus* strains containing the ROSA-like phage may explain the ability of the bacteria to colonize chronic wound tissues and their ability to exist in the commensal state. Interestingly these colonizing strains were exclusively found in uninfected ulcers that represent the niche of these strains. The role played by these bacteria would be directed towards the establishment of colonizing wounds and/or help others pathogens in pathogenic process; the ROSA-like insertion bringing a selective advantage to this role. From a clinical point of view, the stability of phage and the very low potential of virulence of the colonizing strain suggest that it is not necessary to treat ulcers carrying this *S. aureus* type. Our findings may contribute to better diagnosis and improved treatment of diabetic ulcers.
Acknowledgments

Pr. Jean-Philippe Lavigne is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Fer-15 nematodes were provided by the *Caenorhabditis* Genetics Center, a foundation of the NIH National Center for Research Resources (NCRR).

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N.M. researched data and wrote the manuscript. T.K.P. researched data and reviewed the manuscript. G.L. contributed to discussion and reviewed/edited the manuscript. D.O.C. contributed to discussion and reviewed/edited the manuscript. S.J.F. contributed to discussion and reviewed/edited the manuscript. S.A.R. contributed to discussion and reviewed/edited the manuscript. M.B. researched data. C.D.R. researched data and reviewed the manuscript. F.V. contributed to discussion and reviewed the manuscript. A.S. designed the study and reviewed/edited the manuscript. J.P.L. designed the study, researched data and wrote the manuscript.

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Competing Interests

The authors have declared that no competing interests exist.

Abbreviations

BHI: Brain Heart Infusion; CC: Clonal Complexe; DFU: Diabetic Foot Ulcer; LT50: Lethal Time 50%; MSSA: methicillin-sensitive *S. aureus*; MRSA: methicillin-resistant *S. aureus*
References


Figure Legends

Figure 1. Virulence of the different *S. aureus* strains in zebrafish embryos.

A. Survival curves of zebrafish embryos following infection with two infecting (NSA739 and NSA18026) and two colonizing (NSA1385 and NSA1322) strains.

B. Survival curves of zebrafish embryos following infection with NSA739, NSA1385 and NSA1385(P-) strains.

Figure 2. Kinetics of biofilm formation for the different *S. aureus* strains.

Bars represent the standard deviations from three independent experiments with triplicate for each one.
Table 1. 50% Lethal Time of *Caenorhabditis elegans* infected by the different *S. aureus* strains and evaluation of CFU of each strain in the *C. elegans* digestive tract.

The results are representative of at least five independent trials for each group of strains. p: Pairwise comparison using a log rank tests. NS: non significant. LT50: 50% Lethal Time

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics of the <em>S. aureus</em> strain</th>
<th>LT50 in days (IC95% inf-sup)</th>
<th>p</th>
<th>Median CFU [range]/nematode after 72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSA1385†</td>
<td>clinical, strain isolated from colonized</td>
<td>4.3 (4.0-4.6)</td>
<td>-</td>
<td>2.2 x 10^5 [1.0-3.4 10^5]</td>
</tr>
<tr>
<td></td>
<td>DFU* (Grade 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSA1322†</td>
<td>clinical, strain isolated from colonized</td>
<td>4.0 (3.7-4.4)</td>
<td>NS</td>
<td>6.1 x 10^5 [4.9-6.6 10^5]</td>
</tr>
<tr>
<td></td>
<td>DFU* (Grade 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSA739†</td>
<td>clinical, strain isolated from infected</td>
<td>1.7 (1.4-2.0)</td>
<td>&lt;0.001</td>
<td>5.0 x 10^5 [4.8-5.2 10^5]</td>
</tr>
<tr>
<td></td>
<td>DFU* (Grade 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSA18026†</td>
<td>clinical, strain isolated from infected</td>
<td>1.8 (1.5-2.2)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFU* (Grade 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSA1385(P-)</td>
<td>strain isolated from colonized DFU*</td>
<td>4.2 x 10^5 [3.8-4.4 10^5]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Grade 1) after excision of the phage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newman</td>
<td>reference</td>
<td>4.6 (4.4-4.9)</td>
<td>NS</td>
<td>4.9 x 10^5 [4.2-5.6 10^5]</td>
</tr>
<tr>
<td>OP50</td>
<td>control strain</td>
<td>7.8 (7.5-8.1)</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
</tbody>
</table>

*DFU, diabetic foot ulcer
†Sotto et al., 2008 (Ref 4)
Table 2. Distribution of ROSA-like phage in a collection of *S. aureus* isolated in France.

<table>
<thead>
<tr>
<th>Infection/Colonisation</th>
<th>Isolation Site</th>
<th>N°. of strains</th>
<th>N°. of CC8/CC5 strains</th>
<th>N°. of strains with phage ROSA-like insertion</th>
<th>N°. of CC8/CC5 strains with phage ROSA-like insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFI*</td>
<td>120</td>
<td>6</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Acute Cutaneous Infection</td>
<td>10</td>
<td>2</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>SSTI†</td>
<td>9</td>
<td>2</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Bacteremia</td>
<td>8</td>
<td>3</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Endocarditis</td>
<td>8</td>
<td>2</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Pneumonia</td>
<td>20</td>
<td>3</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Cystic fibrosis sputum</td>
<td>11</td>
<td>3</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>Colonization</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nose carriage</td>
<td>131</td>
<td>16</td>
<td>1 (0.8%)</td>
<td>1 (6.3%)</td>
</tr>
<tr>
<td></td>
<td>DFU‡</td>
<td>75</td>
<td>39</td>
<td>39 (52%)</td>
<td>39 (100%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>392</td>
<td>76</td>
<td>40 (10.2%)</td>
<td>40 (52.6%)</td>
</tr>
</tbody>
</table>

*DFI, Diabetic Foot Infection  
†SSTI (skin and soft tissues infections): Cellulitis (n=4), Necrotizing fasciitis (n=3), Abscess (n=2)  
‡DFU, Diabetic Foot Ulcer
Caenorhabditis elegans and zebrafish killing models

In vivo model systems are essential for our understanding of infectious diseases in human host. Non-mammalian models are useful surrogate hosts. These models were used to develop an easy model system of host-pathogen interactions to identify basic evolutionarily conserved pathways associated with microbial pathogenesis.

The nematode leaves in soil and eat environmental bacteria. It is a simple and easy handling model appears of highly interest to evaluate virulence of numerous clinical isolates and notably to study host-pathogen interactions to identify basic evolutionarily conserved pathways associated with microbial pathogenesis. In particular, this has revealed important factors of the host response with remarkable parallels in higher organisms. This organism’s short 2–3-week life span facilitates host-bacteria interaction analysis, offering an ideal compromise between complexity and tractability.

The principle of zebrafish infection assay is equivalent to the nematode model. The difference is the presence of an innate immune system, resembling that of mammals. Moreover the transparency of the embryos and use of fluorescent bacteria make it possible to follow infection in real time. Briefly, bacteria used to infect embryos were grown in 50 ml BHI until they reach optical density at 600 nm of about 1.0 and harvested by centrifugation (4500 g, 10 min). The supernatant was discarded and pellet of cells re-suspended in sterile PBS. Bacterial concentration was determined by serial decimal dilutions and plating on solid media. Zebrafish embryos (30 hpf) were mechanically dechorionated and anaesthetized by immersion in 0.02% (w/v) buffered tricaine. Embryos were embedded in 3% (w/v) methylcellulose. Non-filament glass capillaries were pulled by an electrode puller in order to obtain a fine injection tip. Embryos were injected individually using glass microcapillary pipettes filled with the bacterial suspension of known concentration. Microinjections of 1 nl of bacterial suspension were directed into the bloodstream. To perform the injections, the following equipment was used: a pneumatic micropump (World Precision Instruments PV820), a micromanipulator and a dissecting microscope. After injections embryos were placed in a 96-well microtitre dish (each embryo in a separate well). Each S. aureus strain was injected into a group of 20
embryos. Following infection, embryos were observed for signs of mortality several times a day (up to 120 hpf) and numbers recorded at each time point. Mortality was assumed by heart beat cessation.

Sequencing of the NSA1385 strain and the 44kb insertion

Genomic DNA of \textit{S. aureus} NSA1385 was sequenced using a 454 Life Science-Roche platform by Lifesequencing S.L., Valencia (Spain). The 454 GS Assembler 2.5.3 software was used to assemble 124414 sequence reads into 117 contigs where 78 were larger than 500 bp. The N50 (median) of length of the contig assembly was 82.091 bp and the largest was 174.821 bp. The contigs were linked into 7 scaffolds or super-contigs. The N50 of the length of the scaffolding and the largest scaffold is 2.901.504 bp.

The sequence of the ROSA-lihe phage was:

```
1 tagtttaatt ctgctcattt gagtctaca atttatctct cttctcattat tagtgacagt
gctctcatatt ctactactact ctactactact actactactact
tactactactact actactactact tactactactact tactactactact tactactactact
```

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Detection of phage insertion/deletion

<table>
<thead>
<tr>
<th>Detection</th>
<th>Primers</th>
<th>Mix</th>
<th>Protocol</th>
<th>Length</th>
</tr>
</thead>
</table>
| Phage insertion | rpm-F: 5' TGGCAGTACCAAAGAAAGAAGACTTC
isdB-R: 5' GACACACTCAAAGCCAAAACAATAA | 10X buffer: 5 µL, 1.5 mM MgCl$_2$, 20 pmol of each of the 2 primers, 2 mM each dNTP, 1 U of Taq polymerase, 5 µL of purified DNA | Denaturation: 4 min at 94°C, PCR: 30 cycles of 30 s at 94°C, 30 s at 50°C and 45 s at 72°C, Extension step: 5 min at 72°C | 490 bp  |
Figure. Map similarity cluster using unweighted-pair group method using arithmetic averages (UPGMA) tree of optical maps performed on different *Staphylococcus aureus* strains. The scale indicates the percentage of genetic difference.

![Map Similarity Cluster using UPGMA](image)

CC8-MSSA
Figure. Organization of the phage insertion in PhiNM2 hotspot in *S. aureus* using MAUVE alignment.

(A) Phi-NM2 phage in *S. aureus* Newman; (B) Rosa phage; (C) Rosa-like phage in *S. aureus* NSA1385. Phi-NM2 phage and Rosa-like phage are located upstream the *isd* locus in *S. aureus* genome. Colored outlined blocks surround regions of the genome sequence that aligned to part of another genome. The colored bars inside the blocks are related to the level of sequence similarities. Comparison of the 3 sequences shows a high similarity between Rosa phage and Rosa-like phage in comparison with Phi-NM2 phage. MAUVE alignment also reveals a genome sequence rearrangement between Rosa and Rosa-like phage with sequence inversion in Rosa-like.