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## Existence of a Colonizing *Staphylococcus aureus* Strain Isolated in Diabetic Foot Ulcers



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***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 the genetic basis for the differences in the pathogenic versus colonizing potential of *S. aureus* isolated from diabetic foot ulcers (DFUs). By performing optical map comparisons of a collection of *S. aureus* strains isolated from DFUs, we brought to light a prophage present in noninfecting bacteria. The phage, namely ROSA-like, was localized in a hotspot region  $\Phi$ NM2 near the locus *isd*, the iron surface determinant system. The integrated phage significantly reduces the virulence of the strain and increases the biofilm formation. DFUs seem 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 DFUs, which modulates the relationship of the bacteria with its human host. This phage appears to attenuate bacterial virulence and promote colonization.**

*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 (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 or brain-heart infusion broth.

#### *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

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See accompanying article, p. 2701.

**Table 1—LT50 of *C. elegans* infected by the different *S. aureus* strains and evaluation of CFU of each strain in the *C. elegans* digestive tract**

Strain	Characteristics of the <i>S. aureus</i> strain	LT50 in days (95% CI inf-sup)	<i>P</i> NSA1385 vs. other strains	Median CFU [range] in nematode after 72 h
NSA1385†	Clinical, strain isolated from colonized DFU (grade 1)	4.3 (4.0–4.6)	—	$2.2 \times 10^5$ [ $1.0\text{--}3.4 \times 10^5$ ]
NSA1322†	Clinical, strain isolated from colonized DFU (grade 1)	4.0 (3.7–4.4)	NS	$6.1 \times 10^5$ [ $4.9\text{--}6.6 \times 10^5$ ]
NSA739†	Clinical, strain isolated from infected DFU (grade 3)	1.7 (1.4–2.0)	<0.001	$5.0 \times 10^5$ [ $4.8\text{--}5.2 \times 10^5$ ]
NSA18026†	Clinical, strain isolated from infected DFU (grade 4)	1.8 (1.5–2.2)	<0.001	$5.5 \times 10^5$ [ $4.8\text{--}6.1 \times 10^5$ ]
NSA1385(P–)	Strain isolated from colonized DFU (grade 1) after excision of the phage	1.6 (1.3–1.8)	<0.001	$4.2 \times 10^5$ [ $3.8\text{--}4.4 \times 10^5$ ]
Newman	Reference	4.6 (4.4–4.9)	NS	$4.9 \times 10^5$ [ $4.2\text{--}5.6 \times 10^5$ ]
OP50	Control strain	7.8 (7.5–8.1)	<0.001	—

The results are representative of at least five independent trials for each group of strains. *P* is a pairwise comparison using a log-rank test. †Sotto et al. (4). inf-sup, inferior-superior.

and calculating the LT50 and LT100 (median lethal time 50% and 100%, respectively).

The presence of *S. aureus* in the *Caenorhabditis elegans* digestive tract was determined at 72 h 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 the Supplementary Data.

### Optical Maps

Twenty-two strains from our panel of colonizing and infecting *S. aureus* strains isolated from DFUs (5) were chosen for optical mapping. Optical maps were provided by OpGen (Gaithersburg, MD), prepared on the Argus Optical Mapping System as described previously (9), and analyzed with the support of Phylogene (Bernis, France). The optical maps of the studied strains were then compared with the in silico restriction maps of 19 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 44-kb Insertion

Genomic DNA of *S. aureus* NSA1385 was sequenced using a 454 Life Sciences-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 is presented in the Supplementary Data. After purification, PCR products were sequenced using a PerkinElmer ABI 377 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 Beuzire, France) according to

the manufacturer's recommendations (10). Three experiments with three repeats each (three 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<sub>3</sub> (50  $\mu$ mol/L) 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 colony-forming unit (CFU) and compared with 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 the S-PLUS 2000 software package (Insightful Corporation, Seattle, WA), and results were considered significant at *P* < 0.05.

## RESULTS

### Noninfecting 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 DFUs are less virulent than strains isolated from grade 2–4 DFUs (4). We analyzed the behavior of five clinical strains: the two uninfecting strains (NSA1322 and NSA1385 from grade 1 DFU), the two infecting strains (NSA739 and 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 *Escherichia 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 ( $4.0\text{--}4.6 \pm 0.3$  vs.  $1.6\text{--}1.7 \pm 0.2$  days, 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 uninfected strains (NSA1322 and NSA1385) also exhibit lower virulence in zebrafish embryos. We compared mortality rates over a 92-h period. The two colonizing strains caused less embryo deaths than the two infecting strains ( $P < 0.001$ ) (Fig. 1A and B).

#### Nonvirulent Colonizing *S. aureus* Strains Carry a Genetic Island

To investigate the difference between colonizing and infecting strains isolated from DFUs (4), we used optical mapping to analyze their genome organization (Supplementary Fig. 1). The five colonizing strains were clonal (>99% similarity) and clustered closely to two reference strains (Newman and NCTC8325) with ~98% similarity. The majority of the infecting strains belonged to different clonal groups with <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 a large insertion located exclusively in all of the colonizing strains (CC8- and CC5-MSSA). The insertion was located at the previously described  $\Phi$ NM2 integration hotspot (Supplementary Fig. 2), a known hotspot for genetic insertions, with phage insertions identified in the published

genomes of the two reference strains (Newman and NCTC8325) (14).

#### The 44-kb Genetic Island Corresponds to a ROSA-Like Phage

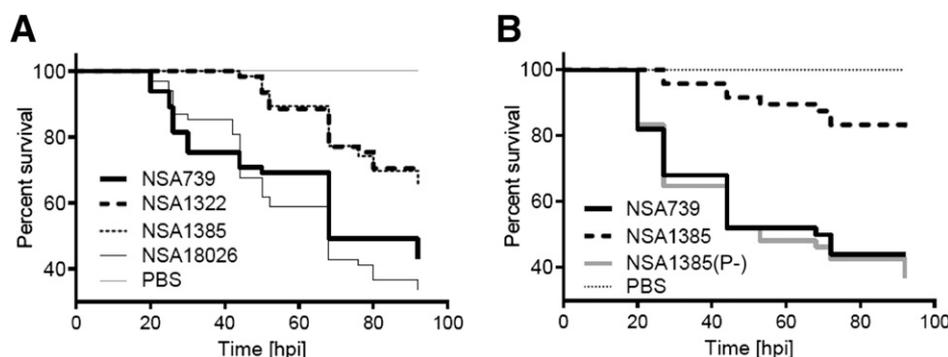
To characterize the genetic insertion present in the colonizing strains, a draft genome sequence of NSA1385 was determined to  $\sim 29\times$  coverage. The integration site is in the intergenic region between *rpmF* (encoding the 50S ribosomal protein L32) and *isdB* (encoding the staphylococcal hemoglobin 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 open reading frames and is allocated within the 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 (Supplementary Fig. 2). We named this genetic island ROSA-like. More information can be found in the Supplementary Data.

#### Genetic Island Is Associated With Colonization of Chronic Wounds in France

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

#### Genetic Island Promotes Biofilm Formation

To determine the impact of the genetic insertion, we studied biofilm formation using the BioFilm Ring Test. In



**Figure 1**—Virulence of the different *S. aureus* strains in zebrafish embryos. **A**: Survival curves of zebrafish embryos after infection with two infecting (NSA739 and NSA18026) and two colonizing (NSA1385 and NSA1322) strains. **B**: Survival curves of zebrafish embryos after infection with NSA739, NSA1385, and NSA1385(P<sup>-</sup>) strains. hpi, hours post-infection.

**Table 2—Distribution of ROSA-like phage in a collection of *S. aureus* isolated in France**

Isolation site		No. of strains	No. of CC8/ CC5 strains	No. of strains with phage ROSA-like insertion	No. of CC8/CC5 strains with phage ROSA-like insertion
Infection	DFI	120	6	0 (0%)	0 (0%)
	Acute cutaneous infection	10	2	0 (0%)	0 (0%)
	SSTI†	9	2	0 (0%)	0 (0%)
	Bacteremia	8	3	0 (0%)	0 (0%)
	Endocarditis	8	2	0 (0%)	0 (0%)
	Pneumonia	20	3	0 (0%)	0 (0%)
	Cystic fibrosis sputum	11	3	0 (0%)	0 (0%)
Colonization	Nose carriage	131	16	1 (0.8%)	1 (6.3%)
	DFU	75	39	39 (52%)	39 (100%)
Total		392	76	40 (10.2%)	40 (52.6%)

DFI, diabetic foot infection. †SSTI (skin and soft tissues infections): cellulitis ( $n = 4$ ), necrotizing fasciitis ( $n = 3$ ), and abscess ( $n = 2$ ).

brain-heart infusion medium, we observed that the colonizing NSA1385 strain formed biofilms more rapidly than the infecting NSA739 strain (150 vs. 180 min) (Fig. 2). These results confirmed the colonizing role of the investigated strains.

#### 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-).

#### Excision of the ROSA-Like Phage Restores Biofilm Formation and Bacterial Virulence

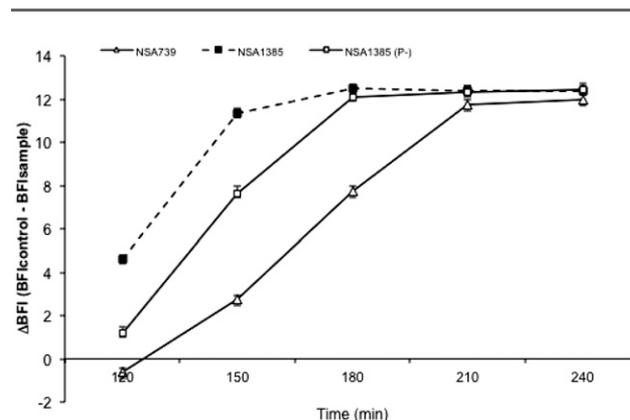
To definitively understand the impact of the phage insertion in the colonizing strain, we evaluated the biofilm formation and the virulence of NSA1385(P-). We observed the restoration of biofilm formation (Fig. 2) and bacterial virulence using the two in vivo models (Table 1 and Fig. 1B). All these data 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 is the most prevalent in DFUs (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 DFUs (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., Pantone-Valentine leukocidin) or resistance determinants (SCC<sub>mec</sub> 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



**Figure 2—Kinetics of biofilm formation for the different *S. aureus* strains. Lines represent the SDs from three independent experiments with triplicate for each one. BFI, BioFilm Formation Index.**

rearrangements noted (compared with 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 toward the establishment of colonizing wounds and/or help other pathogens in the 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.

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

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