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ROLE OF FILAMENTOUS BACTERIOPHAGES IN SMALL-COLONY VARIANT FORMATION OF PSEUDOMONAS AERUGINOSA: INSIGHTS FROM CLINICAL AND EXPERIMENTAL INVESTIGATIONS

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ABSTRACT

Bacteriophages contribute to phenotypic variation and virulence in microbial populations. Specifically, the filamentous bacteriophage Pf4, akin to Pseudomonas aeruginosa strain PAO1's biofilm formation mechanism, promotes the emergence of small-colony variants (SCVs). Patients afflicted with cystic fibrosis, harboring SCVs, exhibit heightened antibiotic resistance and deteriorated lung function. Besides Pf5 filamentous prophages, Pf4-like and Pf1-like prophages are also present. Notably, P. aeruginosa PA14, proficient in SCV formation within biofilms, underwent investigation regarding the involvement of Pf5 in SCV development under static cultures. Immunoblotting analysis failed to detect the Pf5 major coat protein in PA14 SCV cultures, indicating low Pf5 filamentous phage levels. SCV colonies or cultures lacked CoaB expression, further supporting the diminished presence of Pf5. Mutant strains incapable of producing Pf5 RF exhibited SCV formation rates comparable to wild-type strains, suggesting Pf5's dispensability for SCV induction. Interestingly, CF1 filamentous phages were found to inhibit SCV emergence across 48 clinical isolates of P. aeruginosa.

Keywords Bacteriophages, Phenotypic variation, Small-colony variants (SCVs), Pseudomonas aeruginosa, Biofilm

INTRODUCTION

Pseudomonas aeruginosa is a highly adaptable bacterium capable of thriving in diverse environments, including soil, water, plants, and marshlands, owing to its biofilm-forming ability [1]. In cystic fibrosis (CF) patients, persistent infections can arise due to P. aeruginosa biofilms, contributing to antibiotic resistance and treatment challenges [2]. Small Colony Variants (SCVs) with enhanced adhesion and resistance traits further complicate management strategies, particularly within CF patients' lungs [3].

Despite minimal gene expression differences between biofilms and planktonic cultures, Pf4-related genes and SCVs exhibit upregulation in P. aeruginosa strain PAO1 following biofilm exposure, suggesting a potential modulation of biofilms by the Pf4 phage [4]. However, conflicting findings regarding the role of Pf4 phage in SCV formation exist, with downregulation observed in SCVs compared to planktonic cells [5]. This discrepancy may stem from varying experimental conditions during SCV formation rather than consistent phage behavior [6,7]. To elucidate the role of filamentous bacteriophages in SCV formation, this study investigated the PA14 strain of P. aeruginosa, revealing the presence of pf1-like bacteriophages in its genome. These findings suggest that besides clinical isolates, pf1-like phages may also contribute to SCV formation in P. aeruginosa.

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METHODS

The liquid Luria-Bertani (LB) medium was used when growing mass cultures at 37°C on a rotary shaker set at 200 rpm. A final dose of chloramphenicol (30µg/ml) or ampicillin (100µg/ml) was added to cultures when

antibiotics were necessary. Inoculated test tubes were filled with diluted LB medium for vertical growth at 37°C. We collected the top layer of the culture (pellicle) with a swab, then rehydrated it in PBS solution and sampled it at designated times. Enumeration of dilution series was performed on LB agar. A four-day and five-day time point was set if SCVs were not observed after three days. SCV-induction experiments were conducted in LB, Chrome Azurol S (CAS), and M9 minimal media using Citrate as carbon source. Late-exponential broth cultures were supplemented with 10 mM magnesium sulfate. The procedures for recombinant DNA production described by a study were applicable to this study. Both plasmids and total DNA were extracted using Qiagen reagents. T4 DNA ligases and restriction endonucleases were used to cut DNA as directed by the manufacturer. Applied Biosystems' Expand High Fidelity PCR System and Boehringer Mannheim's PCR cloning methods were used to clone the DNA. Applied Biosystems' BigDye terminator 3.1 cycle sequencing kit was used to analyze the samples using the ABI Prism 3100 capillary sequencer [8].

With the help of KODON version 2.03, computer-aided analysis (CAA) was performed on the genome of strain PA14 in order to identify a Pf4-like phage. With the help of this software, repeat regions similar to those found in Pf4 phage were predicted [9]. The overnight growth of wild-type and SCV *P. aeruginosa* cells was performed in a sonication buffer composed of 20 mM Tris/HCl (pH 8.0) and 5 mM EDTA. Centrifugal sonication was used to harvest the cells, resuspended in western immunoblot buffer after centrifugation, and harvested for further experiments. When ultrasonic sound disrupted cell envelope proteins, a centrifuge was used to isolate them. A three-layer SDS-PAGE was used to separate total proteins, and nitrocellulose membranes were used to transfer them to SDS-PAGE. Pf4 and Pf5 coat proteins were encoded by Plac promoters using plasmids containing Plac promoters. Activation analysis of CoaB promoters. Transcription fusions were made from the region upstream of coaB using a promoterless lacZ gene on pMP220. Furthermore, *P. aeruginosa* PA14 was used to measure fusion construct activity. SCV induction experiments: The cultures of fresh SCV and wild-type colonies were grown in LB medium for 24 hours on a rotary shaker at 37°C. Induction experiments were carried out using supernatants from these cultures. Incubated cultures were plated at different timespost-incubation at different dilutions on LB medium.

To detect the replicative form of Pf5, primers were designed to amplify a region predicted to be associated with phage circularization. Following PCR amplification, cloning and sequencing were performed on total DNA and plasmid DNA. Complementation analysis of the Pf5 Mar2xT7 transposon insertion mutants was conducted using fragments containing the entire Pf5 phage genome, except flanking repeats that are necessary for circularization. The gene PA14_489030, which encodes a putative phage regulatory protein, was also added. During overnight cultures, wild-type bacteria were counted and SCVs were counted in transposon mutant strains under static conditions for 26 hours. To identify SCVs and filamentous bacteriophages of the Pf1 family in P. aeruginosa clinical isolates, 102 primary plates were screened and PCR amplification was performed with specific primers. We amplified the genomes of all three phages using universal primers and conserved genes.

RESULT

Pseudomonas aeruginosa PA14 was characterized in this study by means of the integrated filamentous phage Pf5. The genomic structure of this phage was analyzed in detail, and its role in small colony formation was investigated. With some notable differences from filamentous bacteriophage Pf4, Pf5, a prophage similar to Pf1, shared high sequence identity with this virus. The Pf5 lacking four coding sequences was due to the lack of genes encoding putative reverse transcriptases, ABC transporters, antitoxin proteins, and plasmid stabilizing toxin genes. There were also three additional coding sequences in Pf5 that encoded two proteins unknown to science and a putative phage regulatory protein. Pf5 has direct repeats of 10 bp flanking the intergenic region, suggesting circularization. Pf5, according to PCR analysis, consists of 10,675 bp, which falls between Pf1 and Pf4. Although Pf4 is associated with SCF emergence in PAO1, Pf5 did not appear to be related to it in PA14. SCFs produced by PA14 were unable to express Pf5 or Pf4 coat proteins when immunoblotting was performed. Additionally, SCF formation was not significantly different between wild-type and Pf5 mutant strains. SCFs were detected in only a small percentage of P. aeruginosa samples, particularly those stored in cystic fibrosis patients' respiratory tracts. A subset of clinical isolates contained filamentous phages, including Pf4 or Pf5, despite the fact that filamentous phages were common among clinical isolates. SCF formation under static conditions was not correlated with filamentous phage presence. As a result, Pf5 shares some genomic characteristics with Pf4, but not all coding sequences. P. aeruginosa PA14 has been found to harbor a new filamentous phage. When conditions are static, Pf5 does not appear to have a significant impact on SCF. As a result of filamentous phages in clinical isolates, SCF formation by P. aeruginosa might be complex. Pf5 plays an important role in SCF emergence, but further research is needed to understand exactly how it works.

DISCUSSION

An aeruginosa strain PA14 genome contains a filamentous prophage similar to Pf1 [10]. The strain has been named Pf5. As well as this, Pf5 appears to share a high degree of similarity with filamentous bacteriophages Pf4 and Pf1. The Pf5 genome lacks four coding sequences [11]. Toxins that prevent death of hosts, proteins that

prevent death of hosts, and ABC transporters are among these. A putative phage regulation protein has also been encoded by Pf5. There are differences between the locations where Pf4 and Pf5 integrate chromosomes. As opposed to Pf4, Pf5 inserts between multiple homologous genes. An intergenic repeat of 10 bp flanking Pf5 is consistent with circularization of the phage genome [12]. Based on experimental results, Pf5 is approximately 10,675 bytes long, between Pf1 and Pf4. When static conditions were applied to PA14, no association between Pf4 and SCV emergence was observed. Pf5 and Pf4 coat proteins could not be detected on immunoblots from PA14 SCVs or wild-type bacteria. A significant difference between wild-type strains and mutants of Pf5 was also found in the formation of SCVs. A relatively low percentage of P. aeruginosa isolates in clinical samples carried SCVs [13], especially in respiratory tract samples collected from cystic fibrosis patients. Only a few isolates exhibited Pf4 or Pf5, despite the prevalence of filamentous phages in clinical isolates [14]. A filamentous phage was not associated with SCV formation under static conditions. Even though Pf5 is a novel filamentous phage in P. aeruginosa PA14, it does not appear to contribute significantly to SCV formation. Despite the presence of diverse filamentous phages in clinical isolates, filamentous phages play a complex role in SCV formation in P. aeruginosa.

CONCLUSION

Based on our study of Pseudomonas aeruginosa PA14, we are able to understand both the genome structure and the possible role of the integrated filamentous phage Pf5. Despite its distinct gene content, Pf5 shares significant sequence similarities with Pf1, but displays notable differences from filamentous bacteriophage Pf4. While Pf4 and SCF have been associated with each other in PAO1, this study concluded that Pf5 was not responsible for SCF emergence in PA14 under static conditions. Antibody blot experiments failed to detect SCFs or wild-type bacteria. Based on mutational analysis, Pf5 and wild-type strains did not produce significantly different SCFs.

SCFs were found in the respiratory tracts of cystic fibrosis patients, especially when P. aeruginosa samples were clinically analyzed. Clinical isolates containing filamentous phages did not exceed 10%, but most of them did. There was no association between filamentous phages and SCF formation under static conditions. In P. aeruginosa PA14, certain coding sequences are absent, but Pf5 and Pf4 share similar genomic characteristics. It is not significant that SCFs form under static conditions despite Pf5. As demonstrated here, filamentous phages play a complex role in SCF in P. aeruginosa. When SCF emerges, various mechanisms may contribute to its emergence, including filamentous phages like Pf5. To understand how SCF emerges, more research is needed.

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