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Phenotypic variation in the *Pseudomonas fluorescens* clinical strain MFN1032

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Abstract

Pseudomonas fluorescens is a highly heterogeneous species and includes both avirulent strains and clinical strains involved in nosocomial infections. We previously demonstrated that clinical strain MFN1032 has hemolytic activity involving phospholipase C (PlcC) and biosurfactants (BSs), similar to that of the opportunistic pathogen *Pseudomonas aeruginosa*. When incubated under specific conditions, MFN1032 forms translucent phenotypic variant colonies defective in hemolysis, but not necessarily in PlcC. We analyzed eight variants of the original strain MFN1032 and found that they clustered into two groups. Mutations of genes encoding the two-component regulatory system GacS/GacA are responsible for phenotypic variation in the first group of variants. These group 1 variants did not produce secondary metabolites and had impaired biofilm formation. The second group was composed of hyperflagellated cells with enhanced biofilm capacity: they did not produce BSs and were thus unable to swarm. Artificial reduction of the intracellular level of c-di-GMP restored the ability to form biofilm to levels shown by the wild type, but production of BSs was still repressed. Phenotypic variation might increase the virulence potential of this strain.

Keywords: *Pseudomonas fluorescens*; Phenotypic variation; Biofilm; c-di-GMP; Biosurfactants

1. Introduction

Environmental adaptability of microorganisms results from genetic diversity and natural selection. Bacteria also have complex regulatory networks enabling them to colonize a variety of environments [1].

This adaptive behavior can be correlated with phenotypic variation, which is mainly, but not necessarily, influenced by the external environment. One process involved in phenotype diversification is phase variation, which is usually a reversible, high-frequency phenotype switching corresponding to differential expression of one or several genes. Phase variation generates subpopulations within a clonal population. The switch between the various states is generally a stochastic event which can be modulated by external factors [14]. Reversion is a requirement to be considered as phase variation, but it cannot be observed under laboratory conditions [32]. Several studies have described mechanisms involved in phase variation, and although observations of phase variation are increasing, few mechanisms have been reported. These can be divided into genetic rearrangements (DNA inversion or duplication, transposition, homologous recombination, slipped-strand mispairing) or epigenetic modification/regulation [11].

In *Pseudomonas* sp., phenotypic variation has been described for rhizospheric bacteria and the opportunistic pathogen

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Pseudomonas aeruginosa, representative of the genus. Phenotypic variation in crop-protective bacteria *Pseudomonas* was shown to affect mainly the production of exo-enzymes, secondary metabolites and colonization properties [2,26,28,31]. Most of the strains displayed increased motility, generally associated with a longer length of flagellum. These phenotypic variations are correlated with the accumulation of spontaneous mutations in the *gacS* or *gacA* genes [29]. These genes encode the GacA/GacS two-component regulatory system which regulates secondary metabolism, exo-enzyme production and biofilm formation, among other functions. Random spontaneous mutations of the *gacA* and/or *gacS* genes seem to result from a dysfunction in the MutS mismatch repair system, which is negatively regulated by the general stress–response regulator RpoS, itself under the control of the Gac system [30]. These mutations seem to be mediated by site-specific recombinases, although the molecular mechanism has not yet been established [6,19]. The site-specific recombinase is probably responsible for phase variation, and *gac* mutants could be selected *a posteriori*. Phenotypic variations, mainly biofilm-related, frequently occur in the pathogen *P. aeruginosa* as a result of environmental pressure [16]. Small colony variants (SCVs) have also been isolated from the lungs of cystic fibrosis (CF) patients and this phenotype has been associated with the ability of *P. aeruginosa* to persist in the lungs and cause chronic infection [34]. Contrary to rhizobacteria, spontaneous *gac* mutations are not assumed to be involved. By sensing environmental factors, a complex regulatory network controls phenotypic variation (and perhaps the switch between acute infections and chronic persistence) [8]. The GacS/LadS/RetS pathway may play a key role by finely controlling the level of RsmZ and subsequent free RsmA, which plays a critical role in *P. aeruginosa* virulence [21]. SCV emergence is enhanced in *gacS*$^{-}$ PA14 biofilm (which could also correspond to a low level of *rsmZ*), but functional GacS is necessary for the reversion process [4].

A biofilm-related phenotype also seems to be related to the intracellular level of the second messenger c-di-GMP [12]. Although c-di-GMP regulation of biofilm has been reported in diverse bacteria, the mechanism responsible for the switch is not yet understood.

We report the first phenotypic characterization of variants from a clinical *Pseudomonas fluorescens* strain, MFN1032, isolated from a patient suffering from pulmonary tract that we had reported previously [3]. This strain displayed phenotypic variation when incubated at 37 °C. Our objectives were to determine whether these variants enhanced the virulence of this opportunistic pathogen and to identify the mechanism(s) involved.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Strain MFN1032 was isolated from a patient suffering from pulmonary tract infection and identified as *P. fluorescens* biovar I [3]. The bacteria were cultured in Luria Bertani medium (LB) or King B (KB) medium and incubated at various temperatures between 17 and 37 °C in a gyratory shaker at 180 rpm. When necessary, 15 μg/mL gentamicin and 40 μg/mL IPTG were added.

2.2. Measurement of phase variation frequencies

Aliquots of 20 mL of KB were each inoculated with one colony from KB agar plate and the cultures grown for three days, with shaking, at 28 °C or 37 °C. The optical density of the cultures was measured and they were diluted such that when plating on KB medium, an average of 300 colonies per plate were obtained. For estimations of frequency, at least 1500 colonies were counted. The frequency of switching was obtained by dividing the number of switches by the number of generations.

2.3. Extracellular activities

Lecithinase and protease activities were recorded after 48 h incubation at 28 °C on eggyolk and milk agar plates, respectively. Agar plates supplemented with 2% sheep red blood cells (SRBCs) were used to screen for β-hemolytic activity (β-HA) after 24–48 h growth at 28 °C. The plates were examined for enzyme activities on substrates. Opaque zones showed lecithinase activity, whilst protease and hemolytic activities produced clear zones.

2.4. Biosurfactant (BS) analysis

Reverse-phase liquid chromatography coupled with mass spectrometric detection was used to identify BSs. The method was adapted from Morin [20]. When required, a drop-collapse-test was also performed as previously described [25].

2.5. Motility assays, static biofilm assay and quantification

Motility and microarray biofilm assays were performed as described previously [25].

2.6. Electron microscopy

Visualization of flagella was performed as described previously [25].

2.7. Scanning confocal laser microscopy analyses of biofilm

Biofilms were formed on glass microscope slides immersed in 30 mL of LB medium in 9 cm diameter Petri dishes. Cells were labelled with 2.5 μM of Syto 61 Red fluorochrome (Molecular Probes) for 5 min at room temperature and cellulose polymers were stained blue using the calcofluor white fluorochrome (Sigma) (0.3 mg/mL). Slides were washed twice in phosphate-buffered saline and biofilms were observed using a Leica DM6000B confocal microscope (Leica Microsystems, Heidelberg, Germany), with the immersion 63X objective.