Comparative Analysis Of The Development Of Swarming Communities Of Bacillus Subtilis In Case Of Pta And ComXP Mutant Strains

Kassem Hamze, Zaher Zeaiter, Ghayas Lakkis, Ali Shaib, Ahmad Kobaissi

Abstract: Swarming Experiments were carried on with Bacillus subtilis strains to identify the activity of certain genes in the swarming ability and surfactin production. We will examine the effect of comXP as well as pta mutations on the capability of swarming. In different experiments we showed that strain OMG 903 that carries mutation in comXP managed to produce surfactin but showed, attenuated defective and random swarming pattern; strain OMG 928 that carries mutation in pta gene, managed to produce surfactin and showed normal swarming pattern, meanwhile double mutation in comXP and pta in strain OMG 929 lead to the absence of surfactin production and didn't manage Thesetoswarmdatashowed. that a threshold of surfactin production is necessary for a normal swarming pattern.

Index Terms: Bacillus subtilis, swarming, surfactin, acetyl phosphate, pheromone ComX.

INTRODUCTION

Because life is ever moving and nothing is sessile, even the smallest living creatures need to move and translocate to new environment searching for nutrients and escaping stressful surroundings. Starting from this concept, bacteria possessing flagella, when grown over semi-solid surfaces, can adapt their locomotion machinery to achieve a specialized form of flagellum-driven motility called swarming. Swarming migration is preceded by a profound modification of cell morphology, whereby the short planktonic cells differentiate into hyper-flagellated swarm cells. Differentiated cells keep themselves in close cell–cell contact in a monolayer manner (Hamze etal., 2009; Harshey et al., 2003; López et al., 2009; Kearns, D. B. & Losick, R. 2003) and migrate in multicellular rafts along their longitudinal axis. Swarming aspect canvary greatly according to species or even growth conditions starting from medium constituents, humidity, agar percentage and other parameters such as temperature and pH (Julkowska et al., 2005). The swarming phenotype is reversible and swarm cells are known to switch back to their planktonic form when transferred into liquid medium (Harshey, R. M., 2003) which gives them advantage to adapt the environmental changes that might occur on this small micro- environment. Recently, there are quite impressive advances in the study of the regulatory pathways that lead to the understanding of swarming behavior in different bacterial models. It has now become clear that many of these pathways also affect the formation of biofilms, surface attached bacterial colonies. Therefor we can say that decision-making between rapidly colonizing a surface and biofilm formation, which is timing and energy consuming,for example is central to bacterial survival among competitors (Verstraeten et al., 2008). We are studying the swarming of B. subtilis over a fully defined B-medium in a Petri dish or what we call a swarm plate, in which the bacteria migrate from a central inoculum as hyper-branching dendrites, forming radiating patterns covering several square centimeters in a few hours. Following inoculation of the plate with 10^4 cells (that is 2 µL); the bacteria multiply with an estimated generation time of about 90 min (Julkowska et al., 2005). After 11–12 h of growth, the inoculum forms the mother colony (MC), approximately 30 µm thick, and 4 mm in diameter (Fig.1, A). We assume that this growth period is necessary to build a critical mass therefore providing the chemical signal (quorum sensing) that is sufficient to trigger in some cells the ability to form dendrites. The first visible sign of initiation of swarming is the spreading outwards from the edge of the MC of a transparent zone of surfactin. Approximately one hour later, hemispherical 'buds' approximately 800 µm in diameter, appear from the edge of the MC (Fig.1, A). These form the heads (tips) of the rapidly elongating 10-14 primary dendrites. Surfactin production is essential for formation of the pre-dendrite buds and experiments suggest that its presence modifies the surface of the agar gel, presumably by inducing the formation of a thin layer of fluid close to the agar surface. Flagella, whose deployment presumably depends on an appropriate fluid film on the agar surface, are essential for a later stage in the development of the bud and for driving dendrite migration. Importantly, the entire process of bud formation and elongation of the radiating dendrites, up to lengths of 1.5 cm, occurs as a monolayer of cells (Fig.1,B). The cells in dendrites are distributed in an irregular mesh-like organization, including closely packed but clearly separated cells. Dendrites can be divided into two distinct regions, a long stem containing largely non-motile cells, which remarkably are maintained at an overall constant population density, and the extreme 1.5 mm at the tip where the population density increases sharply (Hamze et al., 2011.). This tip region contains hyper-motile cells that we term

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swarmers, which appear to constitute the motor for elongation. Recent studies, reports the presence of swarmers is important for the formation of buds and then subsequently. (Hamze et al., 2011).

Swarming: Molecular signals

Some of the bacterial cellular functions are triggered by extracellular signals that cause target cells to adopt a particular fate or move to specific developmental program. Speaking about differentiation in bacteria, typically involves autocrine signaling in which all cells in the population produce and respond to the same signal. Swarming is triggered by two key regulators ComX and Pta. ComX pheromone is secreted outside the bacteria and later responds to it when it reaches up to certain concentration that is able to activate ComP that has histidine kinase activity. ComP activates transcriptional factor ComA which plays critical role in activation of srf gene resulting in the transcription of a regulon that includes the srf operon, responsible for the synthesis of a surfactant, the cyclic lipopeptide surfactin (Dubnau, 1991). Ultimately, surfactin is produced which will play critical role in swarming and biofilm synthesis (Hamze et al., 2009). Pta (acetyl phosphate) is implicated in the phosphorylation of many bacterial two-component signal transduction systems, as an alternative phospho-donor for ComA (Klein et al., 2007); (Fig.2). Surfactin is a vital element for successful swarming patterns. Surfactin is an amphipathic molecule with varying lengths of a carbon chain (usually C12 to C16), linked to the cyclic heptapeptide. Surfactin is used widely in industry, has potent surface tension and wetting properties. Reduction of surface tension and maintenance of adequate levels of surface water are presumed to be important for migration of the bacteria. However, the precise role of surfactin remains unclear. Surfactin is synthesized non-ribosomally by a large enzyme complex composed of SrfD and three synthetases SrfA, B, C activated by phosphopantetheinyl transferase or Sfp. It has anti-microbial activity as well as can reduce surface tension. (Steller et al., 2004). In liquid culture comX and pta are responsible for (70% and 20% respectively) 90% of surfactin production (Lopez et al., 2009). In this work we will study the effect of these genes on the swarming migration.

MATERIALS AND METHODS

2.1 STRAINS AND GROWTH CONDITIONS.

Bacterial strains used in this study are listed in Table 1. Bacteria were grown with aeration at 37 °C in minimal B-medium composed of (all final concentrations) 15 mM (NH4)2SO4, 8 mM MgSO4 . 7H2O, 27 mM KCl, 7 mM sodium citrate. 2H2O, 50 mM Tris/HCl, pH 7.5; and 2 mM CaCl2 . 2H2O, 1 µM FeSO4 . 7H2O, 10 µM MnSO4 . 4H2O, 0.6 mM KH2PO4, 4.5 mM glutamic acid, 862 µM lysine, 784 µM tryptophan and 0.5 % glucose were added before use (Antelmann et al., 1997). For the thr auxotrophs, both liquid and solid media were always supplemented with 1 mM threonine. Antibiotics were added to plates at the following final concentrations: 5 µg/ml chloramphenicol, 5 µg/ml kanamycin, 100 µg spectinomycin ml–1 and 5 µg/ mlerythromycin plus 12.5 µg/ml lyncomycin.

Table 1. *Strains used in this study*

Strain	Genotype	Reference and Source
168	*trpC2 swrA sfp*	European B. subtilis Genome Consortium
OMG 900	*trpC2 swrA amyE : : sfp+ cat*	Julkowska et al. (2005)
OMG 903	*trpC2 swrA amyE : : sfp+ cat comXP : : spc*	Hamze et al. (2009)
OMG 928	*trpC2 swrA thrC : : sfp+ erm Δpta : : kam*	Derived from Kim et al., (2001)
OMG 929	*trpC2 swrA thrC : : sfp+ erm Δpta : : kam ΔcomXP : : spc*	Derived from Kim et al., (2001)

2.2 CONDITIONS FOR DENDRITIC SWARMING EXPERIMENTS.

For swarming on B-medium, 9 cm swarm plates containing 25 ml medium (0.7% Bacto agar) were prepared 1 h before inoculation with drying restricted to 5 min before inoculation. Cultures for inoculation were prepared in 10 ml B medium inoculated from a single colony on an LB agar plate and shaken overnight at 37ºC. The culture was diluted to OD570 ~0.1 and grown at 37ºC to D570 ~0.2. This procedure was repeated twice and finally the culture was grown to T4 (4 h after the transition from exponential growth). The culture was diluted, and 10^4 bacteria (2 µl) were placed at the center of a swarm plate and incubated at 30ºC (usually at a relative humidity of 70 %).

2.3 IMAGING.

Photographs of swarming plates were taken at the indicated times, either using an Epson 1600 Pro scanner at a resolution above 600 d.p.i. in transparent mode or by digital camera that should be 10 Mega pixels or above to get good resolution. In order to detect the surfactin ring, or at early stages in the swarming process (up to 16–17 h), when the bacteria normally form only a monolayer, plates should be photographed with reflected light, using high resolution camera. Mainly images were captured using Sony 14.1 Mega pixels with dual shots processing, and figures were prepared using Adobe Photoshop software (version Cs5, 2011).

3 RESULTS

3.1 EFFECT OF COMX AND COMP ON B. SUBTILIS MIGRATION

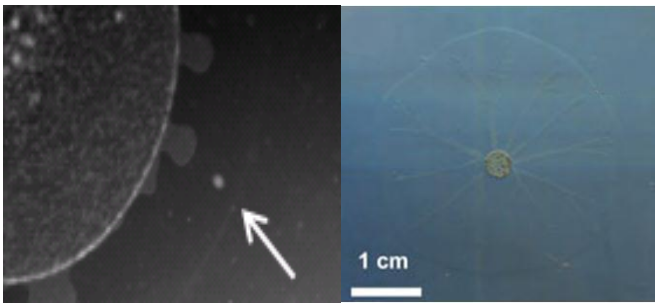
Strain OMG 903, carrying mutation in comXP managed to swarm, but the swarming pattern was different than laboratory strain OMG 900, and more likely instead of producing dendrites, OMG 903 swarmed in unordinary defective random-circular way. We can say that strain OMG 903 can swarm but with different defective new pattern. Strain OMG 903 can fill the plate within the same time needed for strain OMG 900 and taking into consideration that it was growing normally as the control strain. Surfactin deficiency didn't affect the growth or speed of swarming, rather than affecting the pattern of swarming and shape (Fig.3, B).

3.2 Effect of Pta ON B. subtilis MIGRATION

It was necessary to investigate the possible role of pta, to see if such mutation would impair the typical swarming profile, abolish it completely or simply have no effect. Strain OMG 928 (Δpta) defective in the enzyme phosphotransactylase, was therefore tested and showing normal swarming pattern (Fig.3,C) but it is worth to mention that the width of the dendrites in Strain OMG 928 were narrower, Strain OMG 900 had more thick dendrites than strain OMG 928. (Fig. 4, A)

Effect of Pta and ComXP on B. subtilis MIGRATION

Strain OMG 929 that carries mutation in both pta and comXP showed no swarming at all; instead it remained at the center of the plate. Absence of around 90% of surfactin production was critical and the bacteria were not able anymore to swarm (Fig.3,D).



A B

Figure 1: *Swarming patterns of B. Subtilis. (A) swarming pattern of laboratory strain after 12 h of incubation at 30ºC on the synthetic B-medium (0.7% agar). (B) swarming pattern for the same strain after 17h of incubation at 30ºC. The white arrow in (A) and (B) indicates the surfactin 'ring' at the periphery of the swarm, and the black arrow in (A) and (B) indicates the mother colony – the site of the initial inoculation. The dashed white arrow in (A) indicates the Bud.*

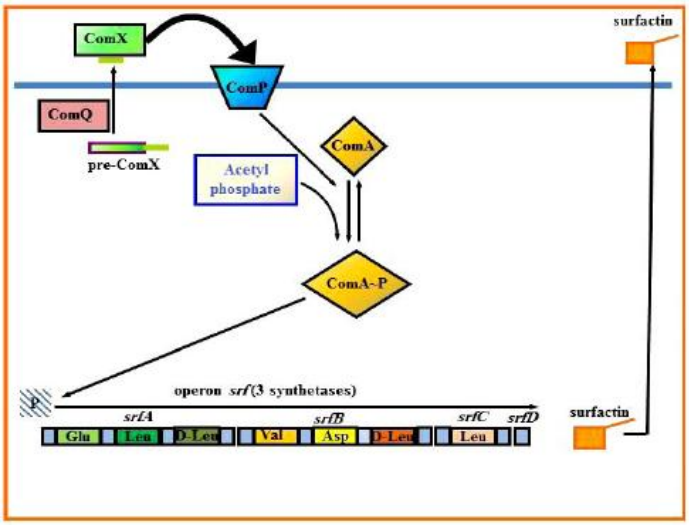


Figure 2. *Scheme of regulatory circuits controlling surfactin production and competence expression in surface cultures. ComAP constitutes a two-component signal transduction system controlled by the interaction of the secreted peptide pheromone, ComX, with the histidine kinase ComP. ComA, also possibly phosphorylated by acetyl phosphate, regulates a large number of genes in addition to srfA. The srfA promoter is subject to multiple regulation and, in addition to the expression of the surfactin synthases (a, b, c) Directs the expression of comS (competence activator) embedded in an alternative reading frame in srfB (not shown in the figure). Following production, surfactin is secreted to the medium probably involving an ABC transporter. Black boxes represent genes. Black arrows and black bars represent positive and negative regulators, respectively. (Hamze et al., 2009)*

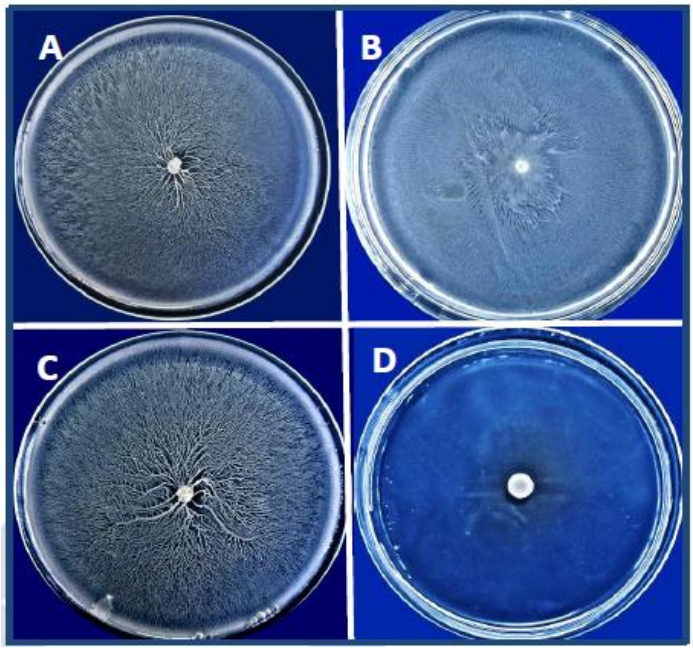


Figure 3: *Swarming results for Bacillus subtilis. (A) Strain OMG 900 show dendritic swarming pattern, (B) strain OMG 903 (ΔcomXP) shows random defective circular pattern. (C) Strain OMG 928 (Δpta) shows swarming pattern that resembles that of OMG 900. (D) strain OMG 929 (ΔcomXP Δpta) shows no swarming at all*

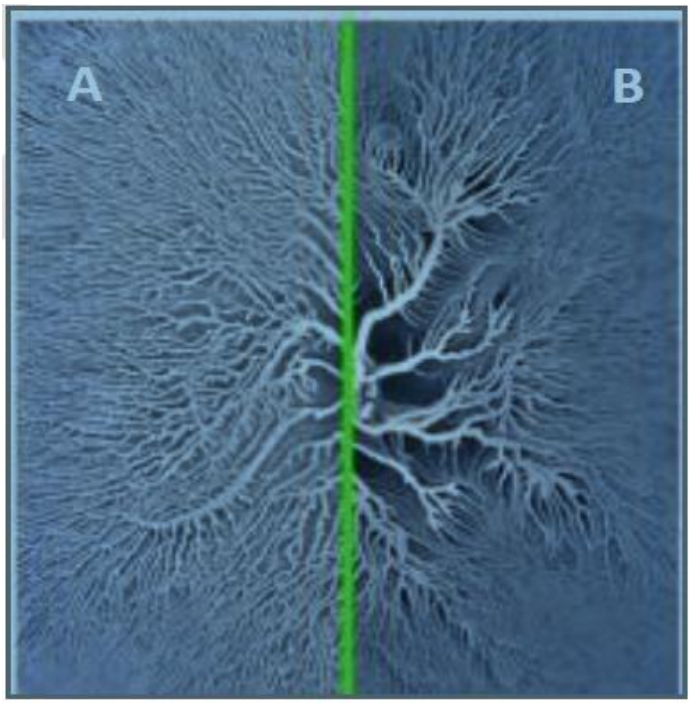


Figure 4: *Dendrites width varies in response to amount of produced surfactin. (A) shows the swarming profile of strain OMG 928, which has dendrites with small diameter compared to (B) shows swarming profile of strain OMG 900, that has more thicker dendrites than strain OMG 928, suggesting that the more surfactin produced, the thicker the dendrites are.*

4 DISCUSSION

Results showed that OMG 903 didn't manage to swarm as OMG 900 did; instead it formed defective random circular swarming patterns (Figure 2. B). This clearly shows that when 70% of surfactin is being missing, due to mutation in comXP, is enough to abolish the well-known dendritic swarming pattern. Hence the idea that whenever there is surfactin there is well defined swarming is wrong. We need certain amount of surfactin for that, let's be on the safe side and say that around 30% of surfactin is not enough for defined dendritic swarming pattern. Meanwhile a mutation in pta (OMG 928), hence impairing acetyl phosphate activity, a predicted 20% decrease in surfactin production didn't affect the swarming profile. In fact the pattern was as that of OMG 900 (Figure 2. C). But it is important to highlight this new idea that the small difference was recorded between the swarming patterns of OMG 900 and OMG 928 lies in the width of dendrites (Figure 3). OMG 900 showed thicker dendrites more than those appeared in OMG 928. This mainly can be referred to the decrease in surfactin production in OMG 928. Such correlation would get clearer if we took into consideration the fact that rivers do not ramifies into smaller streams until the pressure of water becomes lesser. This applies the same on surfactin and dendrites width. By analogy as the more surfactin is found, as more as the dendrites will be thicker, pushing easily their way on the semi-solid medium, and will ramify when this forces that is holding them together weakens. To test what would be the effect of double mutant strain in pta and comXP on swarming, we worked with the double mutant strain OMG 929 (comXP and pta). If we theoretically added the amount produced via comXP with that produced via pta, we notice that about 90% of surfactin production would be missing and only about 10% will be secreted by other unknown pathways. Such approach is important, it enables us to find if such double mutation will lead to impaired swarming as the case in comXP mutant OMG 903 strain or will show total absence of swarming? The results (Figure 2. D) clearly showed total absence of swarming which would be somehow reasonable result, such low amount of surfactin didn't manage to reduce the surface tension and friction to enable the flagella to rotate and move.

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