

## ***In vitro* phase variation studies of *Salmonella Gallinarum* in biofilm formation**

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**Transmission electron microscopy studies were carried out to capture the sequential steps involved in the formation of biofilms *in vitro* by *Salmonella Gallinarum*. Bentonite clay provided in the growth medium acted as a good inert surface for bacterial attachment and biofilm formation at submaximal rates of nutrients. In the initial stages, bacteria started attaching to the surface of bentonite clay and started expressing extracellular polysaccharides (EPS) and curli, which in turn led to repression of flagella and pili. In the later stages, microcolonies were formed leading to maturation of biofilms by overexpression of EPS. The attempt to mimic *in vivo* biofilm formation *in vitro* revealed the process of phase variation.**

**Keywords:** Bentonite clay, biofilms, extracellular polysaccharide, phase variation, *Salmonella Gallinarum*.

BACTERIA do not express the same antigens *in vitro* as *in vivo* – a mechanism known as phase variation. When bacteria grow *in vivo*, they must cope up with a hostile environment in which certain nutrients are not abundant, and where the host attempts to eliminate them in different ways. In such situations, bacteria use certain survival strategies by forming biofilms that are entirely useless when growing *in vitro*, particularly in a ‘favourable’ medium. Moreover, some bacteria produce a thick exopolysaccharide (EPS) capsule that protects them from phagocytosis ([www.exopol.com](http://www.exopol.com)). These structures are lost *in vitro* because, in energy terms, the production of EPS, adhesins, iron-sequestering proteins, etc. is costly and unnecessary. In fact, bacteria sometimes lose genetic information needed to produce these structures, and are never again able to express them<sup>1,2</sup>.

Scientists have recently come to realize that in the natural world, more than 99% of all bacteria exists as biofilms<sup>1</sup>. Rediscovery of a microbiological phenomenon, the ‘biofilms’ exhibited a distinct phenotype with respect to gene transcription and growth rate, where bacteria undergo transition from a planktonic existence to a community-based existence, in which they interact with many neighbours in close proximity<sup>1</sup>. During the formation of biofilms, approximately 40% of genes is altered least twofold<sup>3,4</sup>. Thus, to become

a productive member of a biofilm community, the bacterium must differentiate into a biofilm-associated cell by repressing synthesis of the flagellum that might destabilize the biofilm, and produce EPS that will reinforce the biofilm structure<sup>5,6</sup>.

Fowl typhoid, a septicaemic disease of poultry caused by *Salmonella enterica* subspecies *enterica* serovar *Gallinarum* is difficult to eradicate and may cause chronic infection, which may show the presence of biofilm bacteria. Bacteria forming biofilms *in vivo* will most likely grow on tissue surfaces at submaximal rates of nutrients<sup>7,8</sup>. An *in vitro* technique was designed to grow the bacteria in biofilm mode, by providing bentonite clay as inert surface under nutrient limitation, which simulates natural *in vivo* conditions to express novel proteins<sup>9–11</sup>. Sequential electron microscopy at different stages would provide the much needed information about initial attachment, microcolony formation, maturation and detachment, as schematically represented by earlier workers<sup>12</sup>.

Standard *S. Gallinarum* strain was obtained from the Indian Veterinary Research Institute, Izatnagar, India. The free cells were grown in batch culture at normal concentrations of tryptic soya broth (3.0% TSB) for one day at 37°C and pelleted at 4000 rpm for 10 min at 4°C. The pellet was washed thrice and finally resuspended in PBS and used for transmission electron microscopy (TEM).

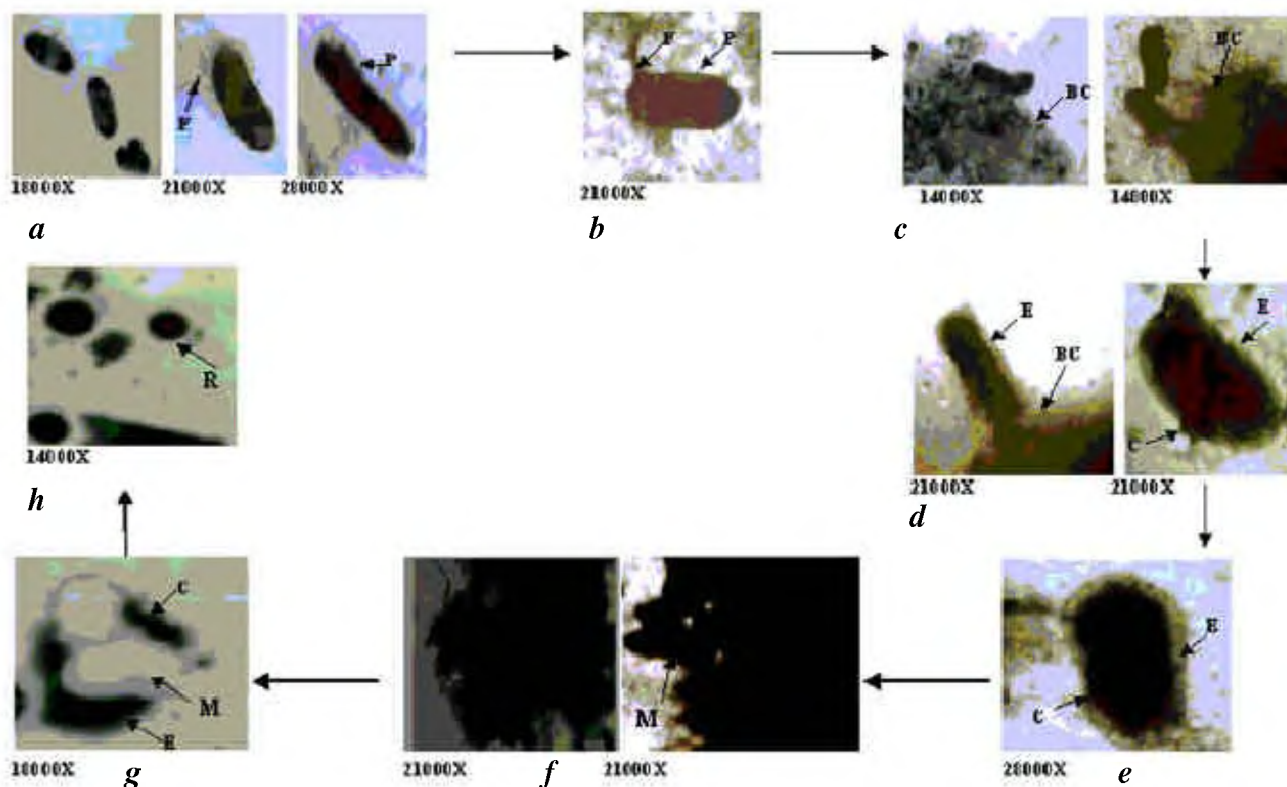
The biofilm cells were grown in submaximal rates of nutrients (0.16% TSB) with bentonite clay (0.3%), and the culture was harvested by discarding the supernatant media to remove any free cells<sup>9,11</sup>. The culture was harvested after one hour, one day, two days and seven days post-inoculation and used for TEM.

For TEM, the standard two-step drop method was followed<sup>13</sup>. A drop of bacterial suspension was placed on para-film and a 200-mesh copper grid (M/s Sigma, USA) with a film reinforced with carbon was placed on the bacterial suspension for 2 min. Excess material was wicked away using filter paper. The grid was then washed with sterile distilled water to reduce the number of adsorbed cells. The coated grid with sample adsorbed to the surface was stained with uranyl acetate (2% aqueous solution) for 2 min. The grids were then air-dried and loaded onto a cartridge and screened under TEM (Joel 100S, M/s Joel Ltd, Japan) under different magnifications.

TEM of *S. Gallinarum* grown in normal broth culture (free cells) had the architecture of rod shape with the presence of appendages such as flagella and pili, and absence of EPS (Figure 1 a).

Cells grown in biofilm mode were freely floating in the initial stages, with presence of flagella and pili as that of free cells. When bacteria got attached to the bentonite clay surface, they triggered the expression of EPS and curli (Figure 1 c–e). On the contrary, the flagella and pili started repressing (Figure 1 d, e). By the end of the seventh day, microcolonies were formed along with maximum expression of curli and EPS (Figure 1 f, g). Production of copious

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**Figure 1.** Transmission electron microscopy study of sequential steps in biofilm formation by *Salmonella Gallinarum*. *a*, Free cells (one day); *b*, One hour post-inoculation (PI); *c*, One day PI (attachment to BC surface); *d*, Two days PI (EPS production); *e*, Two days PI (EPS production); *f*, Seven days PI (maturation and microcolony formation); *g*, Seven days PI (mature biofilms) and *h*, Seven days PI (detachment). BC, Bentonite clay surface; C, Curli; E, Extracellular polysaccharides (EPS); F, Flagella; M, Microcolonies; P, Pili/fimbriae; R, Released cells/free cells.

amounts of these appendages prevented uranyl acetate stain to pass inside the bacteria; thus the bacteria were not stained (Figure 1 *g*). At this stage, we also observed that the cells had lost their architecture by rounding and some cells had detached from the surface (Figure 1 *h*).

Biofilm-forming microorganisms have been shown to elicit specific mechanisms for initial attachment to a surface, microcolony formation, development of a three-dimensional community structure and maturation, and detachment<sup>12</sup>. Micrographs of different steps in biofilm formation by *S. Gallinarum* shown in Figure 1 are in agreement with earlier observations<sup>14,15</sup> and published schematic diagram<sup>12</sup>.

Bacteria approach the surface so close that their motility is slowed down and they form a transient association with the surface. Presence of flagella and pili, and surface-associated proteins are important in attachment to the inert surface<sup>16</sup> (Figure 1 *c*), triggering the expression of EPS and curli (Figure 1 *d, e*). The surface used should be rough, more hydrophobic and coated with surface-conditioning films<sup>17</sup>. The bentonite clay provided, is a colloidal hydrated aluminosilicate ( $\text{Na}_2\text{O} \cdot \text{Al}_2\text{O}_3 \cdot 4\text{SiO}_2 \cdot \text{H}_2\text{O}$ ) earthy powder, which has all the properties required for formation of the biofilm<sup>16</sup>, i.e. it is insoluble in water and organic solvents with a high adsorptive property, and it swells several times its original volume and forms thixotropic gels when small amounts are suspended in water.

After adhering to the inert surface, bacteria become stable for microcolony formation. They begin to multiply while emitting chemical signals that 'intercommunicate' among the bacterial cells. Once the signal intensity exceeds a certain threshold level, genetic mechanisms underlying EPS production (Figure 1 *d, e*) are activated<sup>17</sup>. In this way the bacteria multiply within the embedded EPS matrix, thus giving rise to the formation of a microcolony<sup>18</sup>. During the attachment phase of biofilm development, perhaps after microcolony formation, transcription of specific genes takes place. These are required for the synthesis of EPS. Attachment itself can initiate synthesis of the extracellular matrix in which the sessile bacteria are embedded, followed by formation of water-filled channels. It has been proposed that these channels constitute primitive circulatory system, delivering nutrients to and removing waste products from the communities of cells in the microcolonies.

To become a productive member of the biofilm community, bacteria have to differentiate into biofilm-associated cells by repressing synthesis of flagella (Figure 1 *d, e*), become destabilized and get encased within the EPS (Figure 1 *d, e*). This will reinforce the biofilm structure<sup>19</sup> (Figure 1 *f, g*) as the genes required for synthesis of EPS and curli are upregulated 3- to 5-fold in the recently attached cells vs planktonic counterparts<sup>5,20</sup>, while the genes required for flagellin and pilin synthesis are down-regulated<sup>5,20</sup>. Moreover,

copious amounts of appendages produced at the cell surface, such as curli (Figure 1 *d, e*) could concentrate ionic molecules from the bulk phase as the biofilm develops<sup>15</sup>.

Occasionally, for purely mechanical reasons, some bacteria are shed from the colony, or some bacteria stop producing EPS and are thus 'released' into the surrounding environment (Figure 1 *h*). Biofilm cells may be dispersed either by shedding of daughter cells from actively growing cells, or detachment as a result of nutrient levels or quorum sensing, or shearing of biofilm aggregates (continuous removal of small portions of the biofilm) because of flow effects<sup>21</sup>. As the thickness of EPS increases, anaerobic condition develops within the biofilm with loci of biofilm consisting of anaerobic bacteria. As a result of the combination of film thickness and activity of anaerobic species, the film detaches and sloughs-off from the surface of the substrate<sup>22</sup>. Polysaccharidase enzymes specific for EPS of different organisms, may possibly be produced during different phases of biofilm growth and contribute towards the detachment<sup>23</sup>.

Hence to mimic *in vivo* conditions, *in vitro*, *S. Gallinarum* was grown in liquid medium providing bentonite clay as inert surface, and depleting nutrients and growing for longer periods, which leads to slow growth of bacteria to form biofilms. Biofilm formation was under the process of phase variation, possessing vastly different phenotypic traits, by upregulation of EPS and curli, while flagellin and pilin production was down-regulated.

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## Detection of pathogenesis-related proteins–chitinase and $\beta$ -1,3-glucanase in induced chickpea

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**Pathogenesis-related (PR) proteins, chitinase and  $\beta$ -1,3-glucanase were extracted from induced chickpea plant and purified by gel filtration. Time-course accumulation of these PR-proteins in induced chickpea plants was significantly ( $P = 0.05$ ) higher than the control. Maximum activities of these PR-proteins were recorded after three days of inoculation in all induced plants. Thereafter, the activity decreased progressively. Two chitinases and three  $\beta$ -1,3-glucanases were detected in induced chickpea plants. The molecular mass of the purified chitinases was 31 and 62 kDa and  $\beta$ -1,3-glucanases was 23, 27 and 39 kDa. Purified chitinases**

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