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TRANSMISSION ELECTRON MICROSCOPIC STUDIES ON *IN VITRO* BIOFILM FORMATION BY *ESCHERICHIA COLI* FROM BOVINE MASTITIS CASES

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ABSTRACT

When bacteria grow *in vivo*, they must cope up with a hostile environment in which certain nutrients are not adequate, and where the host attempts to eliminate them in different ways. In such situations, bacteria use certain survival strategies by forming biofilms. In the present study, an *in vitro* technique was designed to grow *Escherichia coli* serotype isolated from bovine mastitis cases, in planktonic mode under nutrient rich condition (3% TSB) and also in biofilm mode, by incorporating bentonite clay (0.3%) under nutrient restricted condition (0.16% TSB). Sequential steps involved in the formation of biofilms *in vitro* by *E.coli* were studied by TEM. *E.coli* grown in planktonic mode had the architecture of rod shape with extracellular appendages. Also, the cells grown in biofilm mode were same as that of cells grown in planktonic mode. In the later stages, bacterium was attached to the surface of bentonite clay. Transition from planktonic state to the biofilm state requires a developmental alteration in flagellae, fimbriae or pili i.e. repression of extracellular appendages and expression of exopolysaccharide leading to formation of microcolonies, a 3-D architecture of mature biofilms (Phase variation).

KEYWORDS

Escherichia coli, planktonic cells, *in vitro* biofilms, Exopolysaccharide, microcolonies

INTRODUCTION

Although mastitis caused by coliforms is of short durations, it may lead to development of chronic infection, which is very difficult to treat [1] and may show presence of biofilm bacteria. When bacteria grow *in vivo*, they must cope up with a hostile environment in which certain nutrients are not adequate, 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 'favorable' medium. Biofilm forming bacteria *in vivo* will most likely grow on tissue surfaces at submaximal rates of nutrients [2, 3]. In the present study, an *in vivo* technique was designed to grow *E.coli* in biofilm mode, by incorporating bentonite clay as an inert surface under restricted nutrient condition, which simulates a natural *in vivo* condition to express novel outer membrane proteins [4, 5, 6]. Sequential steps involved in the formation of biofilms *in vitro* by *Escherichia coli* isolated from the cases of bovine mastitis were studied by transmission electron microscopy (TEM).

MATERIAL AND METHODS

Preparation of culture

Escherichia coli O9 serotype, which was isolated from the cases of bovine mastitis, typed at Central Research Institute, Kasauli and maintained at the Department of Veterinary Microbiology, Veterinary College, Bangalore was used. Free cells were grown in batch culture at normal concentrations (3%) of tryptic soya broth (TSB) for 16 hours at 37°C [5] and centrifuged at 4000 rpm for ten min at 4°C. The pellet was washed thrice and finally resuspended in PBS and used for TEM.

The biofilm cells were grown in nutrient restricted conditions or submaximal rate of nutrients (0.16 % TSB) incorporated with 0.3 % bentonite clay [5]. The culture was harvested after one day, two days, five days and seven days post-inoculation by discarding the supernatant medium to remove any planktonic cells. This culture was used for TEM.

Procedure of transmission electron microscopy

The standard two step drop method as described by [7] was followed for TEM using the facility available at Indian Institute of Horticultural Research, Hesarghatta, Bangalore.

1. A drop of bacterial suspension was placed on paraffin. A 200 mesh electron microscopic copper grid (M/s Sigma, USA) with a film reinforced with carbon was placed on bacterial suspension for 0.5 to 2.0 minutes and excess material was wicked away with an edge of filter paper.
2. Grid was washed with sterile distilled water to reduce the number of adsorbed microorganisms.
3. The coated grid with sample adsorbed to the surface was floated on a drop of 2% uranyl acetate stain for 0.5 to 2.0 min; excess stain was wicked away with a piece of filter paper.
4. The grids were air dried for 1 to 3 minutes, loaded on to cartridge and screened under transmission electron microscope (Joel 100S, M/s Joel Ltd, Japan).

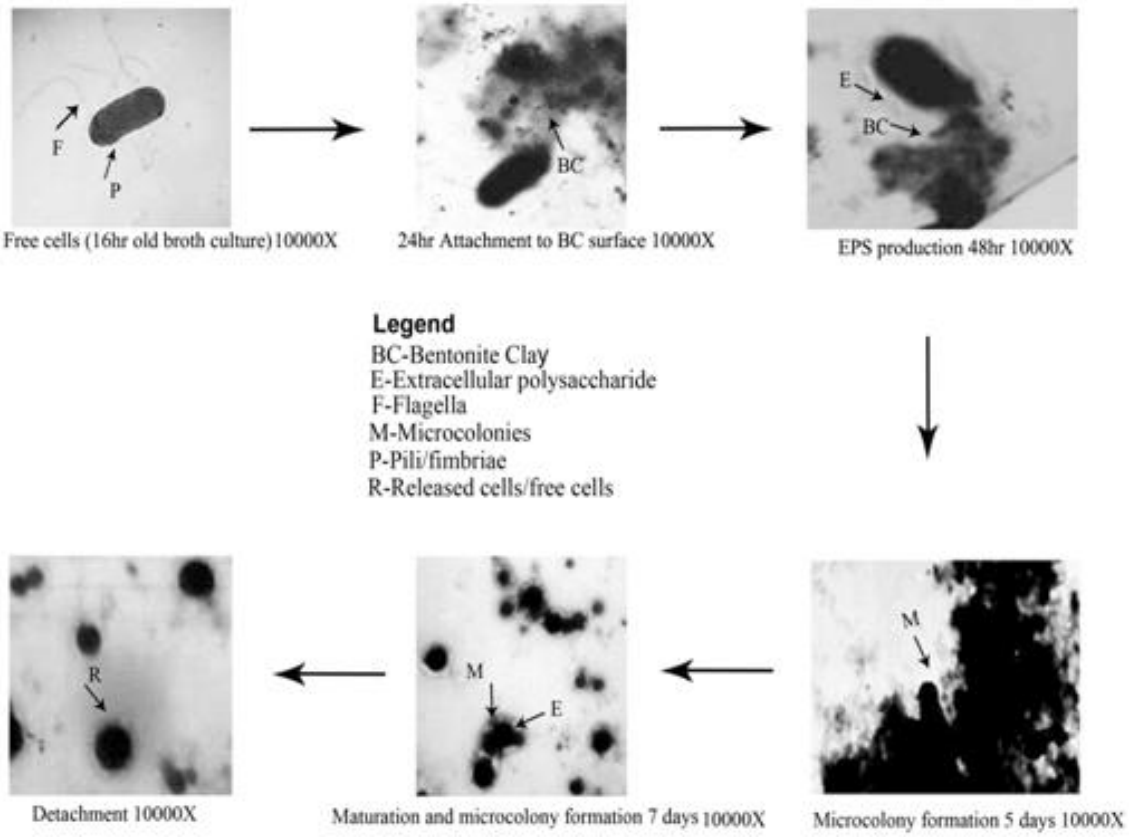
RESULTS

Transmission electron microscopy of sequential steps of biofilm formation by *E.coli* O9 serotype is set out in the Fig. 1.

1. Planktonic cells grown in 3.0 percent TSB for 16 hrs had the architecture of rod shape with the presence of extracellular appendages such as flagella and pili. Also, the cells grown in biofilm mode were freely floating in the initial stages, with presence of flagella and pili as that of cells grown in planktonic mode.
2. **Attachment:** At 24 hrs, organisms grown under BF mode were getting attached to surface of bentonite clay. At 48 hr, the bacterial cell had attached to the surface of bentonite clay, also showed the complete loss/repression of extracellular appendages like fimbriae and flagella and expression of exopolysaccharide/glycocalyx layer around the organism.
3. **Microcolony formation:** On day five, organisms were encased within the EPS leading to formation of microcolonies. On day seven, copious amount of extracellular polysaccharide secretion was seen around the microcolonies.
4. **Maturation:** On day seven, complete covering of organism with extracellular glycocalyx, a light amorphous thin slime around the dense bacilli was seen.
5. **Detachment:** In some, Cell rounding by changing the architecture from rod shape due to detachment from BF or the cells, which are not attached to the surface of bentonite clay.

Fig. 1

Transmission electron microscopy of sequential steps involved in the biofilm Formation



DISCUSSION

In the present study, an attempt was made to delineate different predicted phases of biofilm formation by *E.coli* O9 isolated from cases of bovine mastitis by transmission electron microscopy (Fig. 1). Biofilm forming organisms have been shown to elicit specific mechanisms for initial step of attachment,

microcolony formation wherein organisms were encased within the EPS matrix and maturation and detachment – the final step wherein organisms were getting detached from the surface of bentonite clay. These steps of BF formation by *E.coli* are in agreement with the observations of [8, 9, and 5]. The biofilm formation by *V.cholerae* [10], *P.aeruginosa* [11] involves initial attachment to a solid surface and formation of microcolonies that differentiates into EPS encased mature biofilm[11].

Bentonite clay was incorporated in the media to provide an inert surface, since *E.coli* is known to possess pili or fimbriae and is involved in attachment, not only to animal cells[12] but also to inert surface. Once temporary contact is established, the organisms use either flagella or type IV pili to move along the contact surface in two dimensions, until other bacteria are encountered. They begin to multiply while emitting chemical signals that intercommunicate among the bacterial cells. Once the signal intensity exceeds a certain threshold level, the genetic mechanisms underlying EPS production are activated [11]. Then the bacteria multiply within embedded EPS matrix, thus giving rise to the formation of microcolony[13]. The free cell differentiates into biofilm-associated cell by suppressing the mechanism of synthesis of flagella and gets encased with exopolysaccharide that will reinforce the biofilm structure[10].

In the free-swimming state, flagella and fimbriae are necessary for motility and initial adherence to a solid surface. However, their synthesis is inhibited in biofilms[14 and 15], as such appendages may impede the maintenance of biofilm structure by breaking or inhibiting other surface associations required for communal stability[16]. The number of cells colonizing the inert surface increases when the surrounding media is nutrient restricted. The increased persistence observed in the biofilm cell population could be explained by the fact that biofilm exopolysaccharide matrix traps the available nutrients and promotes the sustained growth of the biofilm microcolonies. In addition, when surrounded by a nutrient-poor medium, bacteria preferentially adhere to a surface capable of adsorbing and concentrating the available nutrients onto itself, thus assuring that the adherent bacteria have maximum access to nutrients[17]. Solid surface provides a resting place as well as concentrating nutrients to the film there upon by *E.coli* as reported by[18 and 19]. Bentonite clay provided, is a colloidal hydrated aluminosilicate (Na_2O Al_2O_3 $4.SiO_2$ H_2O) earthy powder, which has all the properties required for formation of the biofilm[20], *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.

Occasionally, some bacteria are shed from the colony, or some bacteria stop producing EPS and are thus 'released' into the surrounding environment. 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 [21]. 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[22]. Polysaccharidase enzymes specific for EPS of different organisms, may possibly be produced during different phases of biofilm growth and contribute towards the detachment [23].

CONCLUSION

Maturation or transition from planktonic state to the biofilm state requires a developmental alteration in flagellar, fimbrial and EPS synthesis, promoting the resultant three-dimensional architecture of mature biofilms (Phase variation). Hence, to mimic *in vivo* conditions in vitro, *E.coli* serotype O9, isolated from bovine mastitis case was grown

in liquid medium under depleting nutrients, and using bentonite clay as an inert surface. Transmission electron microscopic studies confirmed the growth of *E.coli* serotype O9 under biofilm mode.

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