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EXPERIMENTAL ARTICLES

Adhering Ability of *Stenotrophomonas maltophilia* Is Dependent on Growth Conditions[1]

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Abstract—The growth conditions are known to influence the bacterial adhesion to different kinds of surfaces. In the present study the adhering ability of *S. maltophilia*, on growth in nutrient rich media (Tryptic Soy Broth (TSB)) and minimal media (Luria Bertani (LB)) was checked by viable cell count and spectrophotometric method. TSB grown *S. maltophilia* showed higher adhesion compared to bacteria grown in LB broth, to both biotic and abiotic surfaces. However, when bacteria were grown in LB broth supplemented with different concentrations of glucose, under aerobic conditions, the bacteria grown at lower glucose concentration (2 gm/l) showed maximumadhesion to abiotic surfaces (polystyrene microliter plate) compared to biotic surfaces (mouse trachea, mouse tracheal mucus and HEp–2 cells line). Maximum adhesion to biotic surfaces was seen with cells grown at 4 gm/l of glucose concentration. On the contrary if the cell was grown under microaerophilic conditions maximum adhesion to abiotic and biotic surfaces was achieved with bacteria grown at 1 gm/l and 2 gm/l of glucose concentration respectively. A negative correlation was observed between glucose concentrations and pH of media, the latter declined faster under microaerophilic conditions as compared to aerobic condition.

Keywords: glucose, adhesion, aerobic, microaerophilic, *Stenotrophomonas maltophili*.

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Stenotrophomonas maltophilia is being reported with increasing frequency as an important nosocomial pathogen. It is an opportunistic pathogen colonizing patients in intensive care settings, especially those with underlying debilitating conditions such as immunosuppression, malignancies, and implantation of foreign devices [1–5]. Bacterial adherence is the first step in the pathogenesis of infections of mucosal surfaces or prostheses. *S. maltophilia* strains of both clinical and environmental origin have been reported to adhere to abiotic [4, 6, 7] and biotic surfaces [8].

In a recent study from our laboratory, adherence of *S. maltophilia* has been shown to be due to the involvement of flagella in this process [8]. Earlier studies have demonstrated that exposure of bacteria to altered growth conditions may effect the flagellin expression, which may indirectly affect the adhesion process [9]. Since participation of flagella in the biofilm formation has been documented earlier, it becomes imperative to study the expression of flagella under different growth conditions. It is important as biofilm mode of growth in case of *S. maltophilia* has been suggested to be a virulence factor for this organism especially in relation to respiratory tract.

The expression of virulence genes is controlled by various environmental and host conditions, including temperature, osmolarity, pH, and oxygen and the presence of certain nutrients such as sugars or ions in the bacterial habitat [10, 11]. Milenbachs et al. (1997) found that several readily metabolized sugars, such as glucose, fructose, and ceilobiose, caused down regulation of certain virulence genes [12]. It is known that the catabolism of added sugars might change the pH of the medium, resulting in the suppression of some proteins and enhancement of others [10]. Hence growth of the organism in different media may influence the surface appendages that may be important for bacterial adhesion. Earlier studies on this aspect have focused their attention on a variety of microorganism [13]. But this information with respect to *S. maltophilia* is lacking in literature. Hence, we investigated the effect of supplement media on the adhering ability of *S. maltophilia* to different kinds of biotic and abiotic surfaces, on growth under aerobic and microaerophilic conditions.

MATERIAL AND METHODS

Bacterial isolate. Clinical isolate of *S. maltophilia* (Sm2) was used in this study. Bacteria were preserved by lyophilization and were routinely cultured at 37°C

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on Luria Bertani agar plates. Subcultures were made every week.

Growth conditions. *S. maltophilia* (Sm2) was grown overnight in separate flasks of LB broth supplemented with different concentrations of glucose (0.5, 1, 2, 4, 8 and 16 gm/l) at 37°C. The flasks were incubated under aerobic as well as under microaerophilic condition. The cell pellet was obtained by centrifugation (5000 *g* in 20 min at 4°C), washed twice with PBS (0.01 M, pH 7.2) and resuspended in sterile PBS (0.01 M, pH 7.2) to achieve a bacterial concentration of 10^7 cfu/ml. Bacterial growth thus obtained was checked for its ability to adhere to different kinds of surfaces.

Animals. BALB/c mice of 6–8 weeks old weighing 20–25 gm were procured from central animal house of Panjab University, Chandigarh. Animals were kept in clean polypropylene cages and fed on standard antibiotic free diet (JBD agencies, Pvt. Ltd., India). The study was conducted following approval from the animal ethics committee of Panjab University, Chandigarh. India.

Crude mouse tracheal mucus (MTM) Preparations. 20 μl of sterile PBS (pH 7.2) was repeatedly passed through mouse tracheal lumen to wash out tracheal mucus. The slightly turbid mucus suspension was pooled, centrifuged (1000 *g* for 10 min) and filtered using millipore filter (0.2 λm) to remove cells and debris.

Assay of bacterial adherence to tracheal mucus. Microtiter plates (Nunc, Denmark) were coated with crude mucus at a concentration of 100 ug/ml [14]. Bacteria grown in presence of different concentrations of glucose or oxygen were added to each well and plates were incubated at 37°C for 1 h to check the ability of *S. maltophilia* (Sm2) to adhere to mucus. The wells were washed five times with PBS. The adherent bacteria were desorbed with Triton X–100 (0.05%) and plated for enumeration by plating different dilutions on Luria Bertani plates. All the experiments were conducted in triplicate.

Adherence to mouse trachea (MT). For adherence assay, trachea was excised from BALB/c mice and cut in to pieces of 4 mm in length under sterile conditions. The tracheal pieces were put in small petri dishes, covered with respective bacterial cells (10^7 cfu/ml) and incubated at 37°C for 2 h. After incubation each piece of tracheal tissue was rinsed gently with PBS (0.01 M, pH 7.2), three times to remove unbound bacteria. Each piece was homogenized separately in 1 ml PBS and 100 μl was serially diluted and plated in duplicate on Luria agar plates. The bacterial number was quantitated after overnight incubation at 37°C [8].

Cell Line and Adhesion Assay. Adhesion of *S. maltophilia* to HEp–2 cell line was performed according to the method described by Cravioto et al. (1979) [15] and modified by Lillehoj et al (2002) [16]. Briefly cells were cultured for 20–24 h in 24 well microtiter plate containing an inoculum of 2×10^5 cells per well. Monolayers were made by growing cells in Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal calf serum, 10 mM L–glutamine at 37°C in 5% CO_2. The monolayers were washed three times with phosphate buffered saline (PBS), pH 7.2. HEp-2, cells were incubated with respective bacteria (10^7) in 0.5 ml/ well for 1 h at 37°C and washed three times with PBS. The infected HEp–2 cells were lysed with PBS–0.5% Triton × 100, diluted 10 fold and plated on LB agar to estimate the number of adhering bacteria.

Adhesion assay to polystyrene microtiter plates. Bacterial aliquots (200 μl) of standardized inoculum (10^7 cfu/ml) were added to the wells of sterile flatbottom polystyrene tissue culture plates and incubated at 37°C either for 4 or 24 h to check the adhesion of *S. maltophilia* on growth in presence of different concentrations of glucose/oxygen. The medium was then discarded, and non adherent cells were removed by washing three times with sterile PBS (0.1 M, pH 7.2). Quantitatiation of adhering bacteria was performed both by viable cell enumeration as well as by spectrophotometric method as previously described by Bonaventura et al. (2004) [17], with minor modifications. For plate counts, adherent bacteria (biofilms) were removed from microtiter wells by scraping and then vortexed vigorously and counts estimated by plating serial dilutions of the suspension. For the other method, briefly, slime and adherent organisms were fixed by incubating them for 30 min at 60°C and then stained with Hucker crystal violet (0.4%) for 5 min. After thorough washing with water to remove excess stain, the plates were dried for 30 min at 37°C. The extent of biofilm was determined by measuring the absorbance of stained adherent film upon treatment with acetone:ethanol (30 : 70) at a wavelength of 492 nm [18].

Statistical analysis. All data represent mean values and standard error of at least three replicas of independent experiments. The differences between test and control were analyzed by using student's *t* test. One way ANOVA was applied to check the significant differences among all cases. Correlation coefficient was also applied to check the relationships. Origin 8.0 version software was employed to perform these calculations and plot graphs. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of Incubating Media on the Bacterial Adhesion to Biotic and Abiotic Surfaces. *S. maltophilia* was

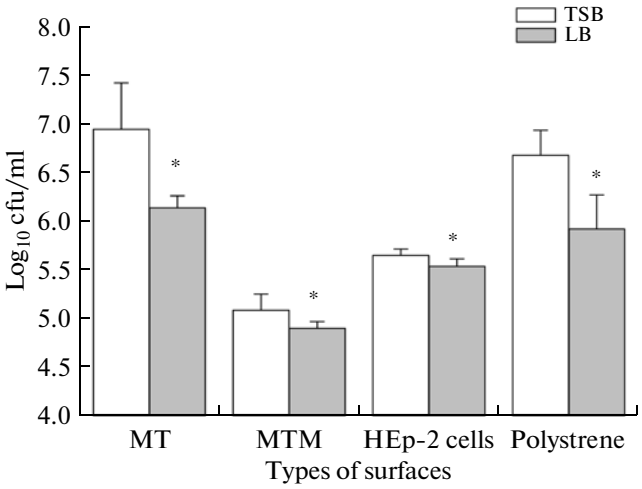

Fig. 1. Log_{10} cfu/ml of adherent bacteria to different kinds of surfaces. *S. maltophilia* was grown in Tryptic Soy Broth (TSB) and Luria broth (LB), the adhering ability of this isolate was checked to mouse trachea (MT), mouse tracheal mucus (MTM), HEp-2 cells line and polystyrene microtiter plates. Significant difference.

incubated over night at 37°C either in TSB or LB broth and checked for adhesion to different kinds of biotic and abiotic surfaces. The results showed significant difference in their ability to adhere to all kinds of biotic and abiotic surfaces. *S. maltophilia* grown in TSB showed higher adhesion to all the surfaces as compared to LB grown bacteria (Fig. 1) and it was maximum in case of its adhesion to mouse trachea followed by polystyrene.

Ability of *S. maltophilia* to adhere following growth in lb supplemented with different concentrations of glucose under aerobic condition. The results showed that different concentrations of glucose showed an impact on bacterial adhesion to different surfaces under aerobic condition. Fig. 2 shows that addition of LB with 4 gm/l glucose gave maximum adhesion to biotic surfaces such as MT, MTM and HEp-2 cells line. Where as adhesion of *S. maltophilia* to polystyrene microtiter plates was best achieved at 2 gm/l of glucose concentration. Ability to adhere was checked on the basis of number of viable adherent bacteria by determining viable count as well as by spectrophotometric method. Similar results were seen by employing both the methods.

The effect of microaerobic condition on the adhering ability of *S. maltophilia* grown in lb broth supplemented with different concentrations of glucose. The results obtained with *S. maltophilia* (Sm2) under microaerophilic condition were similar to that obtained with cells grown under aerobic condition. However the concentration of glucose which gave maximum adherence was lower in case of *S. maltophilia* grown under microaerophilic conditions (Fig. 3). This figure shows that addition of LB with 2 gm/l glucose gave maximum adhesion to biotic surfaces such as MT, MTM and HEp-2 cells line. Where as adhesion of *S. maltophilia* to polystyrene microtiter plates was best achieved at 1 gm/l of glucose concentration under microaerophilic condition.

It was seen that aerobic conditions favored adhesion of *S. maltophilia* to different surfaces compared to microaerophilic condition (Fig. 4). Thus difference in adherence was significant ($p < 0.05$).

effect of bacterial growth on pH. When *S. maltophilia* was grown in presence of different concentrations of glucose and in aerobic and microaerophilic conditions, pH was monitored at regular intervals, pH declined when the concentration of glucose increased, but this decline was significantly lower on incubation in microaerophilic environment than the decline observed under aerobic condition (Fig. 5).

DISCUSSION

In the present study presence of glucose in the medium was found to help bacteria in its ability to adhere to both abiotic and biotic surfaces under aerobic as well as microaerophilic conditions. Earlier studies have also shown that different carbon sources can affect the expression of various virulence factors by altering either growth rate, metabolic pools or pH [19, 20]. However, the adhesion was glucose concentration dependent under both aerobic and microaerophilic conditions. The optimum adhesion process under aerobic condition was seen at a concentration 4 gm/l (to biotic surfaces) and 2 gm/l (to polystyrene). Where as on growth under microaerophilic conditions the same was achieved at half the glucose concentration compared to incubation under aerobic condition. A further increase in glucose concentration was found to be detrimental for the adhesion process. The negative relation between glucose and pH after overnight incubation may be responsible for the decreased adhesion observed on further increasing the glucose amount in the medium. Increased acidity was observed under microaerophilic conditions compared to aerobic conditions. This may be due to an earlier observation that under low oxygen conditions, bacterial cell is stimulated to consume more glucose in aerobic metabolism resulting in production of high acidity [13].

The results of this study are in line with the observation made by Jaradat and Bhunia (2002) [13]. Since adhesion of *S. maltophilia* to biotic and abiotic surfaces has been shown to be mediated through flagella [8, 21], it is likely that under low pH conditions the expression of these bacterial appendages is inhibited. It has been reported that pH of the medium does affect the expression of virulence genes in different organisms [13]. As adhesion to any surface helps in the colonization by the organism, which is an initial step in the bacterial pathogenesis. These results suggest that low pH of the medium has a bearing on the virulence potential of *S. maltophilia*. This may also explain that

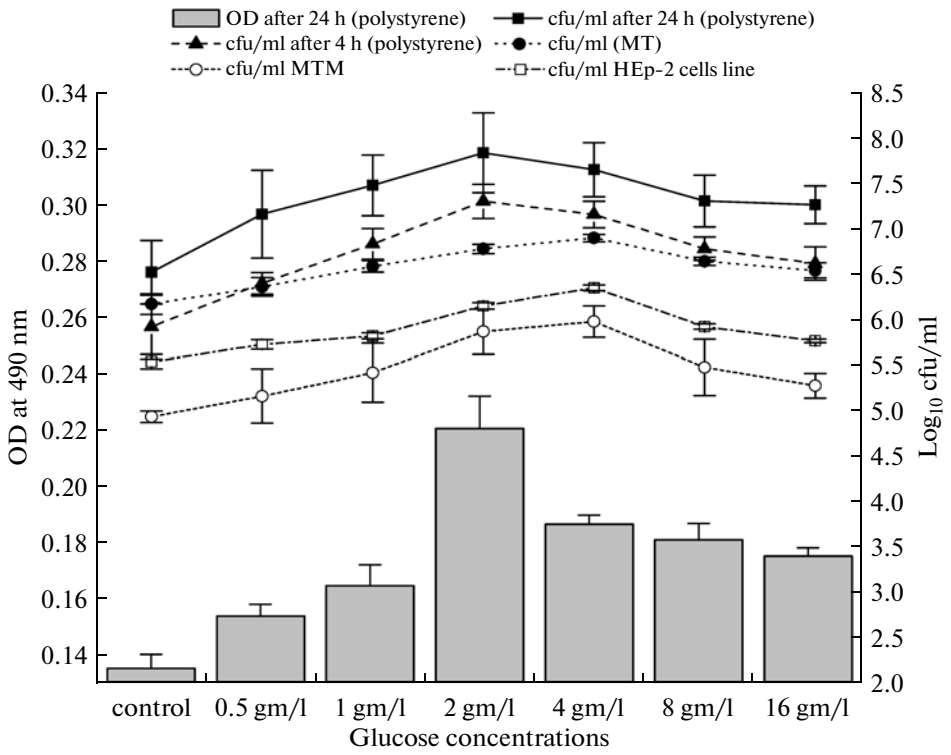

Fig. 2. Effect of glucose supplementation on the adhering ability of *S. maltophilia* under aerobic condition. Figure presents Log_{10} of adherent bacteria (cfu/ml) to mouse trachea (MT), mouse tracheal mucus (MTM), HEp-2 cell line and polystyrene microtiter plates.

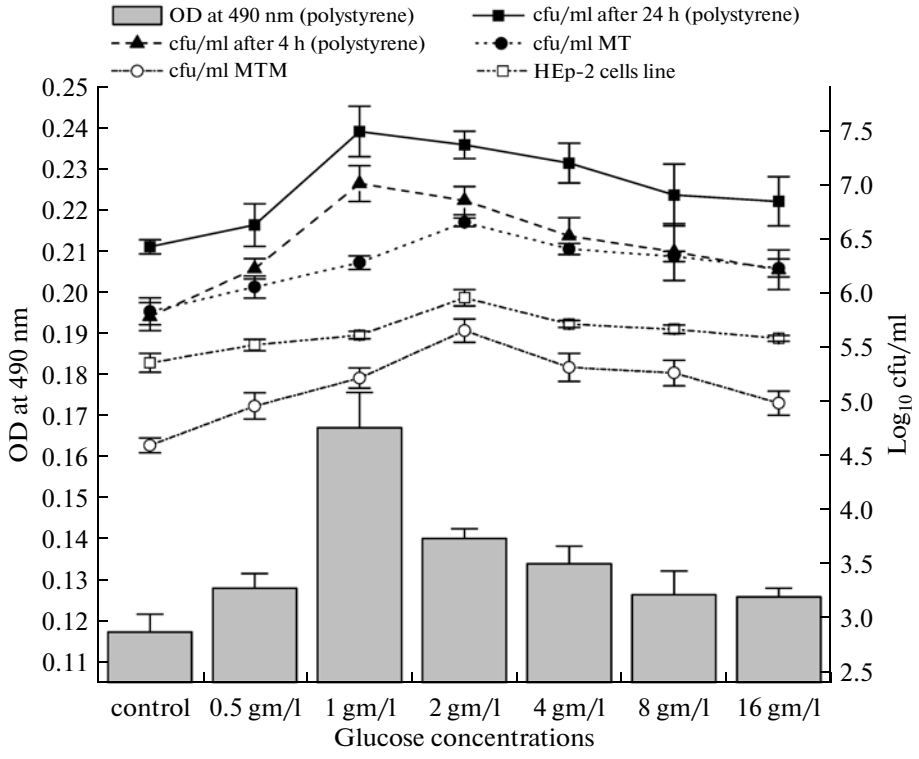

Fig. 3. Effect of glucose supplementation on the adhering ability of *S. maltophilia* under microaerophilic condition. Figure presents Log_{10} of adherent bacteria (cfu/ml) to mouse trachea (MT), mouse tracheal mucus (MTM), HEp-2 cell line and polystyrene microtiter plates.

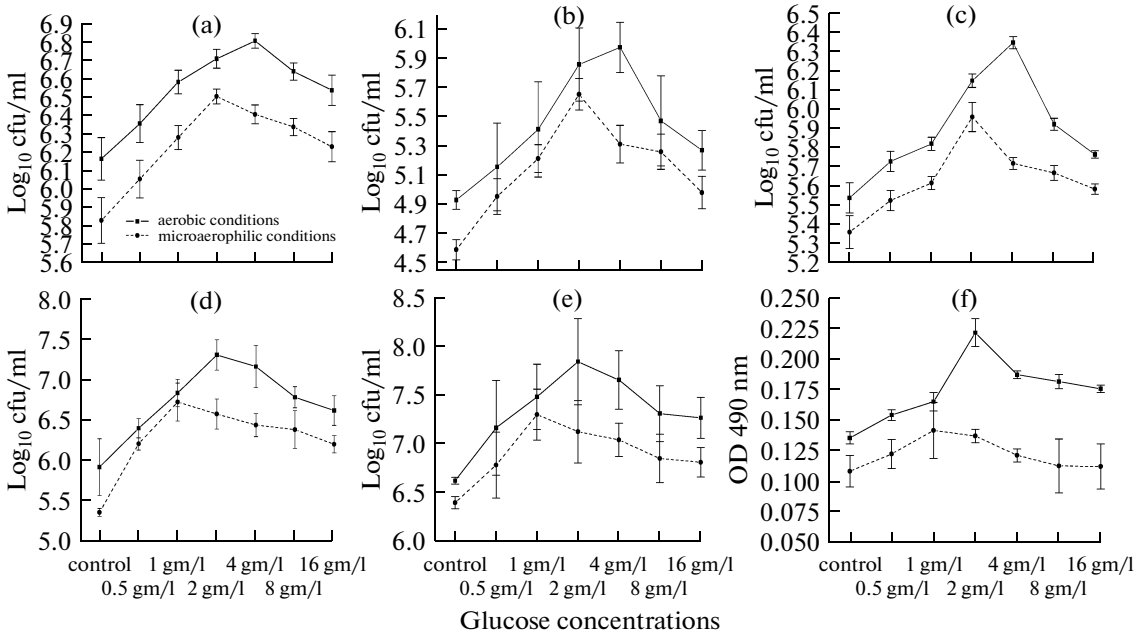

Fig. 4. *S. maltophilia* adhesion to mouse trachea (MT) (a), mouse tracheal mucus(MTM) (b), HEp-2 cells line (c) and polystyrene microtiter plates after different times (cfu/ml after 4 h (d), cfu/ml after 24 h (e) and OD at 490 nm after 24 h (f)) in vitro. The ability of bacteria to adhere when grown under aerobic incubation was significantly higher than micraerophilic incubation significantly.

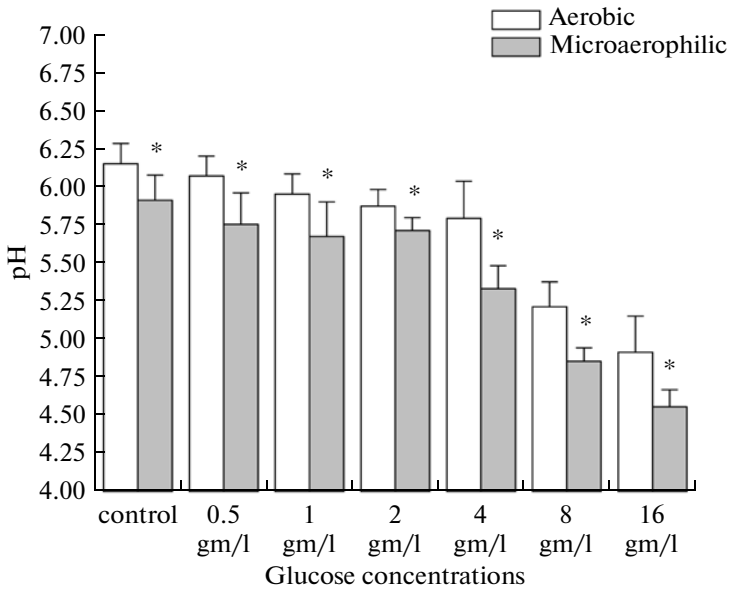

Fig. 5. pH values of *S. maltophilia* growth at 37°C overnight in media containing different concentrations of glucose under aerobic/microaerophilic conditions. The negative relationship was detected between the glucose concentration and pH values in both cases (correlation coefficient: −0.97 and −0.96 respectively). Significant difference in pH values was found between aerobic and micraerophilic condition ($P < 0.05$). Significant difference.

this organism does not cause infections of gastrointestinal tract, where it is likely to be subjected to low pH condition. The results of this study have shown that it is important to select optimum growth conditions for studying the adhesion of *S. maltophilia* to both biotic and abiotic surfaces.

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