Adherence of *Burkholderia pseudomallei* Cells to Cultured Human Epithelial Cell Lines Is Regulated by Growth Temperature

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Received 14 May 2001/Returned for modification 16 July 2001/Accepted 27 September 2001

We have investigated the adherence of *Burkholderia pseudomallei*, cultured under a number of different conditions, to six human epithelial cell lines. While several complex medium compositions had relatively little effect on adherence, growth at 30°C was found to significantly increase adherence to all cell lines relative to that of cultures grown at 37°C (*P* < 0.001).

Meliodosis is a disease of humans and animals, the etiologic agent of which is *Burkholderia pseudomallei*. The major region of endemicity encompasses Southeast Asia and northern Australia, though this is considered to be expanding (8, 10, 11, 24, 25). *B. pseudomallei* is a gram-negative saprophytic bacterium which can frequently be found inhabiting the soil and water of regions of endemicity (9, 35). While infections acquired from the environmental reservoir via percutaneous inoculation have been documented and this is considered the major mode of infection, the roles of inhalation and ingestion are less clear (7–9, 17). Person-to-person or animal-to-person transmission of melioidosis is considered to be rare (7–9). Disease presentation is diverse, including chronic and acute forms of localized and disseminated infection, with the lungs, liver, and spleen being the most commonly affected organs (25). Melioidosis is associated with a high fatality rate and is a major cause of death from community-acquired bacteremic pneumonia in regions of endemicity (7, 23).

Adherence of pathogens to host surfaces is a prerequisite step in the pathogenesis of almost all infectious diseases. Bacterial adherence requires the specific interaction of bacterial molecules, termed adhesins, with host cell membrane molecules or extracellular matrix proteins (2, 16). A modification of this typical interaction is employed by the attaching and effacing enteropathogens which insert the bacterial protein Tir into host cell membranes for use as the receptor for the bacterial adherence intimin (21). Disruption of the adhesin-receptor interaction in many paradigm systems, by mutagenesis or competitive inhibition, resulting in a nonadherent phenotype, is typically associated with a significant reduction in virulence of the pathogen (16, 26, 36). Conversely, the elucidation of specific roles of individual adhesins is complicated by redundancy attributable to the presence of multiple adhesins, as is the case with many species, for example * Bordetella pertussis* (29).

A significant effort has been put into preliminary characterization of *B. pseudomallei* interactions with eukaryotic cells, in particular the ability of *B. pseudomallei* to survive within mammalian phagocytic cells. It has been shown that *B. pseudomallei* has the ability to survive within polymorphonuclear leukocytes (12, 19, 27), macrophages (13, 14, 19, 27), and nonphagocytic cell lines (14, 19). Similar interactions with *Acanthamoeba* have also been described (18). Recently, *B. pseudomallei* has been reported to cause mammalian cell fusion (22), an apparently novel phenotype among those displayed by bacterial pathogens. Relative to the interest in intracellular survival, the preceding steps in pathogenesis, including adherence to host surfaces, have received scant attention. There has been one report of adherence of *B. pseudomallei* to human pharyngeal cells, from which it was concluded that *B. pseudomallei* displayed low attachment ability (1). Despite this, intrabronchial inoculation of mice with *B. pseudomallei* resulted in significant bacterial loads in the lungs, indicating that *B. pseudomallei* has the ability to adhere to the respiratory tract in vivo (1).

Given the importance of adherence to host surfaces in microbial pathogenesis, we decided to investigate the adherence of *B. pseudomallei*. It was our intention to clarify this situation by developing an in vitro model of adherence using the standard methodology of allowing bacteria to adhere to epithelial cell lines in culture, as opposed to primary cells shaking in suspension (1).

**Identification of the *B. pseudomallei* adherent phenotype.** In order to see if *B. pseudomallei* was capable of adhering to epithelial cells, a number of variables were tested. This was to consider the possibility that any potential adherent phenotype could be regulated by growth conditions or possibly be specific for different types of cells. We decided to test adherence to six epithelial cell lines, each derived from different tissues. The cell lines A549, NC1-H292, HEP-2, KB, Chang, and ME-180 were derived from alveolar, bronchial, laryngeal, oral, conjunctival, and cervical tissues, respectively. *B. pseudomallei* strain 08 (6) cultures were grown in Luria-Bertani (LB) medium containing high salt, low salt, and high salt-low iron concentrations at temperatures of 30 and 37°C. All assays were conducted in triplicate with the nonadherent *Escherichia coli* strain DH5α as a negative control (32). The statistical significance of the differences between the means was assessed using a one-way analysis of variance performed with the Bonferroni post hoc test. The results from these experiments (Fig. 1A) show that *B. pseudomallei* grown at 30°C adheres to each cell line significantly more than *B. pseudomallei* grown at 37°C or DH5α (*P* < 0.001). While the levels of adherence to each...
While there was some variation in the degree of adherence displayed by each strain, in each case the 30 and 37°C phenotypes paralleled that seen with strain 08 (results not shown).

To examine whether this apparent increase in the adherence of *B. pseudomallei* grown at 30°C relative to adherence with growth at 37°C was an artifact, *E. coli* DH5α grown at these temperatures was tested for adherence to ME-180 cells. Adherence of cultures grown at 37 and 30°C was found to be 1.33% ± 0.09% and 1.04% ± 0.08%, respectively. Similarly, strain E264 of the closely related but relatively avirulent species *Burkholderia thailandensis* (3, 4) was grown at 37 and 30°C and the degrees of adherence to ME-180 cells were 4.76% ± 0.33% and 4.61% ± 1.21%, respectively. This is potentially another important difference between these species, in addition to the previously demonstrated difference in virulence (3, 4). These results clearly demonstrate that the adherence phenotypes displayed by *B. pseudomallei* grown at 30 and 37°C are not an artifact of the methods employed in this investigation. The apparent temperature regulation of adherence could be the reason why a previous study found *B. pseudomallei* to adhere to pharyngeal cells only at low levels (1).

All of the preceding experiments were conducted with stationary-phase cultures, and even though cultures grown at 30°C were of an optical density equivalent to that of those grown at 37°C at the time of assay, it was decided that any potential effects of the growth phase should be identified at each temperature. The results shown in Fig. 1B clearly demonstrate that bacteria from stationary-phase 30°C cultures adhered significantly more than logarithmic-phase 30°C cultures and both logarithmic- and stationary-phase 37°C cultures ($P < 0.001$). A modest increase in adherence was observed in the logarithmic-phase 37°C cultures in comparison to the stationary-phase 37°C cultures. Taken together, with no information regarding the molecular basis of the demonstrated phenotypes, adherence would appear to be regulated in a complex manner.

Characterization of temperature effects on *B. pseudomallei* adherence. The fact that *B. pseudomallei* cells grown at 30°C adhered significantly more to epithelial cells than those grown at 37°C led us to consider the optimal growth temperature for bacterial adherence to ME-180 cells. Cultures were therefore grown at 5°C intervals from 22 to 37°C. As can be seen from the results shown in Fig. 2A, the optimal temperature for adherence appeared to be 27°C.

The temperature regulation of adherence is interesting when the following is considered. Bacteria grown at 37°C do not show appreciable adherence to host cells, whereas bacteria grown at 30°C do show significant degrees of adherence, and these bacteria are adhering during incubation at 37°C. We therefore investigated the effect of incubation with ME-180 cells at 37°C by performing a time course experiment, in order to see if the incubation at 37°C progressively decreased the levels of adherence. This involved quantifying adherence following incubation for up to 8 h during the assay itself. The results for the 30 and 37°C cultures indicate that levels of adherence progressively increased with incubation time (Fig. 2B), presumably due to the longer period, which allowed attachment and increases in bacterial cell numbers. Given this result, adherence assays were then performed using cultures grown to stationary phase at 30°C, followed by incubation at 37°C for defined lengths of time, before use in a standard 2-h incubation adherence assay. The results shown in Fig. 2C...
clearly illustrate that, following growth at 30°C, an incubation of 15 min at 37°C prior to conducting the assay is enough to cause a significant reduction in adherence (P < 0.005). These results are not due to a decrease in bacterial viability following the temperature shift from 30 to 37°C (results not shown). The mechanism for the reduction of adherence manifested following a temperature shift in LB medium from 30 to 37°C could involve a requirement for de novo protein synthesis for the maintenance of adhesins, assuming adhesins are not synthesized at 37°C in LB medium. Indeed, it has been demonstrated that *Haemophilus influenzae* adherence requires de novo protein synthesis (31). To test this hypothesis, we incubated strain 08 cultures grown overnight at 30°C with tetracycline (10 µg ml⁻¹) at 30°C and without tetracycline at 37°C for 2 h prior to assaying adherence. The results for these experiments were 10.01% ± 1.07% for the tetracycline culture and 11.93% ± 2.97% for the 30-37°C temperature-shifted culture, which were both significantly different from the result for the 30°C control culture without tetracycline (50.00% ± 3.92%, P < 0.001). This indicates that de novo protein synthesis is required for *B. pseudomallei* adherence. In addition, the similarity of results for protein synthesis inhibition at 30°C and the temperature shift to 37°C could indicate that adhesin molecules are not synthesized at 37°C.

The temperature shift experiments described above present an interesting point: bacteria grown at 30°C and incubated at 37°C in LB medium without epithelial cells show a significant reduction in adherence, whereas those incubated at 37°C with epithelial cells in cell culture medium during the assay maintain high levels of adherence. To address this, another temperature shift experiment was devised to uncouple any potential influence of the cell culture medium from the epithelial cells on adherence. Cultures used in the assay were grown at 30°C to stationary phase, pelleted by centrifugation, resuspended in cell culture medium (McCoy’s 5a medium), and incubated at either 30 or 37°C for 4 h prior to adherence assays. Respectively, these results were 91.65% ± 21.19% and 31.96% ± 2.94%. Comparison with the results shown in Fig. 2C indicates that incubation of 30°C-grown stationary-phase bacteria at 37°C in cell culture medium allows for a substantial maintenance of adherence relative to incubation in LB medium (P < 0.005). Incubation of 30°C-grown stationary-phase bacteria in cell culture medium at 30°C also appeared to increase the levels of detectable adherence to ME-180 cells when compared to that of bacteria from 30°C stationary-phase cultures grown in LB medium. Hence, the regulation of adherence appears to involve signals in addition to growth temperature. In *Pseudomonas aeruginosa*, the type III secreted protein, ExoS, has been shown to be induced in response to growth in cell culture medium (33), and it is perhaps likely that *B. pseudomallei* adhesins are similarly induced.

The relevance to pathogenesis of a highly adherent phenotype only when bacteria are grown at temperatures below 37°C certainly merits discussion. It is especially noteworthy that *B.

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**FIG. 2.** The effect of temperature on *B. pseudomallei* adherence to ME-180 cells. (A) Adherence of *B. pseudomallei* strain 08 cultures grown at 22, 27, 32, and 37°C. (B) Adherence of *B. pseudomallei* strain 08 cultures to ME-180 cell monolayers following incubation times ranging from 15 min to 8 h. (C) Adherence of *B. pseudomallei* strain 08 to ME-180 cells following a temperature shift. *B. pseudomallei* strain 08 was grown at 30°C to stationary phase before being incubated at 37°C in LB medium for defined lengths of time prior to assaying adherence to ME-180 cells. Percent adherence was determined by dividing the number of adherent bacteria by the inoculum and multiplying by 100.
pseudomallei is an environmental bacterium, and infection is almost exclusively acquired from soil and water rather than from infected animals or humans. Taking into account the temperature regulation of adherence indicated by these experiments, we propose that this may be important for initial adherence to the host following an encounter from the environmental reservoir. A similar temperature regulation of adherence is shown by *Bordetella bronchiseptica* (28). In this case, the relative increase in adherence of bacteria grown at the lower temperature is less than that seen with *B. pseudomallei*, due mainly to the fact that *B. bronchiseptica* displays relatively high levels of adherence when grown at 37°C (28). Importantly, this temperature regulation of adherence in *B. bronchiseptica* is reflected by colonization levels in vivo, suggesting an important role in overcoming initial clearance mechanisms (5). The precise temperatures of typical environmental reservoirs of *B. pseudomallei* are unknown, and as such the potential relevance of such regulation is speculative. A major paradigm of virulence gene regulation is the ToxR regulon of *Vibrio cholerae*, and this system is influenced by a number of environmental stimuli, including temperature (30). Included in the ToxR regulon are the toxin coregulated pilus and accessory colonization factors (30). During in vitro growth of classical biotype strains in LB medium, the growth temperature for optimal expression of the regulon is 30°C (30), though this is not considered to be relevant to natural infections. Hence, it remains a formal possibility that the temperature regulation of adherence described herein is simply an artifact of in vitro culture of *B. pseudomallei*.

**Qualitative investigation of *B. pseudomallei* adherence using microscopy.** In order to confirm both that *B. pseudomallei* strain 08 was indeed adhering to epithelial cells and that the levels were increased in cultures grown at 30°C, monolayers were Giemsa stained (pH 6.7) and examined by light microscopy. These experiments were conducted using ME-180 cells grown on coverslips. Light microscopy indeed confirmed that bacteria were adhering to cells and that they were far more prevalent in cultures grown at 30°C (Fig. 3). Typically, bacteria grown at 30°C could often be seen clustered together in microcolonies, adhering to one or a few adjacent epithelial cells, while many neighboring epithelial cells in the monolayer were left relatively free of bacteria. Observation of the adherence of

![Image](http://iai.asm.org/)

FIG. 3. Digital images recorded from light microscopy of *B. pseudomallei* strain 08 adhering to ME-180 cells. All samples were stained with Giemsa (pH 6.7) and examined with a 100× objective. (A and B) *B. pseudomallei* bacteria cultured at 30°C until stationary phase prior to the adherence assay. A large number of adherent bacteria can be observed in adherent microcolonies. (C and D) *B. pseudomallei* bacteria cultured at 37°C until stationary phase prior to the adherence assay. Few adherent bacteria can be observed. Bar, 10 μm.
FIG. 4. Scanning electron microscopy of *B. pseudomallei* strain 08 bacteria adhering to epithelial cell lines. (A) Electron micrograph of *B. pseudomallei* adherence to Chang cells after incubation of bacterial cultures at 30°C until stationary phase, demonstrating bacterium-host and bacterium-bacterium interactions. Magnification, ×3,000. (B) Electron micrograph of *B. pseudomallei* adherence to A549 cells after incubation of bacterial cultures at 30°C until stationary phase, demonstrating extensive bacterium-host and bacterium-bacterium interactions. Magnification, ×3,600.
bacteria grown at 37°C showed that while the number of bacterium-cell contacts was considerably lower, adherent microcolonies were essentially absent compared with that seen in bacteria grown at 30°C. At the incubation time used (2 h), there was no consistent and obvious cytopathic effect common to cells with adherent bacteria when compared to uninfected controls. Scanning electron microscopy was utilized to investigate the possible existence of any specific structural features associated with the adherence of B. pseudomallei to epithelial cells. Adherence to all six previously tested cell lines was investigated, and no outstanding structural feature on the surface of the epithelial cells was associated with bacteria-epithelial cell contacts. Likewise, no bacterial structure could be seen mediating attachment to the epithelial cells or to other bacteria. The microcolony formation of adherent bacteria observed was consistent with the results recorded with light microscopy. Epithelial cells associated with adherent microcolonies, in many instances, were associated with many bacteria independent of bacterium-bacterium interactions, and it was apparent that these interactions were intimate (Fig. 4). This illustrates the potential importance of both bacterium-epithelial cell and bacterium-bacterium interactions in the adherence of B. pseudomallei to epithelial cells. It is noteworthy that mechanisms of microcolony formation of other pathogens may involve bacterial as well as host molecules (15, 20).

Concluding remarks. This study clearly shows that B. pseudomallei is capable of adhering efficiently to epithelial cell lines. It would appear that the adherent phenotype is regulated in a complex manner by growth temperature, medium composition, and growth phase and that it is dependent on de novo protein synthesis. The development of an animal model which tests preliminary steps in pathogenesis is necessary to test whether this temperature regulation of adherence is important in the pathogenesis of melioidosis. This investigation provides a basis on which to begin the molecular characterization of the bacterial molecules responsible for the initial interactions with host cells as well as the bacterium-bacterium interactions. Since any adhesins are likely to be regulated, experiments involving expression in heterologous hosts may prove challenging. Molecular characterization of the regulatory mechanisms will also be of interest, as will the characterization of the host molecules required for attachment to B. pseudomallei.


*Editor: V. J. DiRita*