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FEMS Microbiology Letters 252 (2005) 243–249

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

The *Burkholderia pseudomallei* RpoE (AlgU) operon is involved in environmental stress tolerance and biofilm formation

Sunee Korbsrisate [a,*], Muthita Vanaporn [a], Phansupa Kerdsuk [a], Wannapa Kespichayawattana [b], Paiboon Vattanaviboon [c], Pornpimon Kiatpapan [d], Ganjana Lertmemongkolchai [e]

[a] *Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand*
[b] *Laboratory of Immunology, Chulabhorn Research Institute, Bangkok, Thailand*
[c] *Laboratory of Biotechnology, Chulabhorn Research Institute, Bangkok, Thailand*
[d] *Biochemistry Unit, Faculty of Science, Rangsit University, Bangkok, Thailand*
[e] *Department of Clinical Immunology, Faculty of Associated Medical Sciences, Khon Kaen University, Thailand*

Received 17 February 2005; received in revised form 8 August 2005; accepted 1 September 2005

First published online 15 September 2005

Edited by M. Schembri

Abstract

Burkholderia pseudomallei, the causative agent of melioidosis, can be isolated from soil and water. To persist, adapt and survive within and outside their human host, bacteria rely on regulatory mechanisms that allow them to respond rapidly to stressful situations. We have examined the possible role of *B. pseudomallei* alternative sigma factor σ^E (RpoE) in the stress response and found that *rpoE* and its putative regulators (*bprE-rseB-mucD*) are transcribed in a single transcriptional unit. Inactivation of the *rpoE* operon changed the *B. pseudomallei* phenotype. Changes included increased susceptibility to killing by menadione and H_2O_2, susceptibility to high osmolarity, reduced ability to form biofilms, and reduced survival in macrophage J774A.1. Therefore, we conclude that *rpoE* controls gene expression that contributes, at least in part, to *B. pseudomallei* adaptation to adverse environmental conditions.

Keywords: *Burkholderia pseudomallei*; RpoE (AlgU); Oxidative and osmotic stresses; Biofilm; Intracellular survival

1. Introduction

Burkholderia pseudomallei is a Gram-negative bacterium and is the etiological agent of melioidosis. The disease is endemic throughout south-east Asia and northern Australia [1]. The bacterium is a saprophyte found in soil and water, and transmission occurs through direct contact via cuts and abrasions or by inhalation. The incidence of clinical melioidosis is estimated to be 3.6–5.5 cases per 100,000 per annum in the north-eastern part of Thailand [2]. At present, there is no vaccine available. Although there are many studies on virulence factors [3], little is known about the genes involved in stress tolerance by this bacterial pathogen.

To persist, adapt and survive within and outside their human host, bacteria rely on regulatory mechanisms that allow them to respond rapidly to stressful situations. The responses in Gram-negative bacteria such as *Escherichia coli* can be localized in cytoplasmic and extra-cytoplasmic compartments and are controlled by distinct alternative sigma factors (RNA polymerase

* Corresponding author. Tel.: +66 2 418 0569; fax: +66 2 418 1636.
E-mail address: grsks@mahidol.ac.th (S. Korbsrisate).

0378-1097/$22.00
doi:10.1016/j.femsle.2005.09.002

subunits). The well-characterized cytoplasmic response is co-ordinated by σ^{32} [4]. The extracytoplasmic response, in contrast, is less well defined and is controlled by at least three partially overlapping signal transduction systems including the CpxRA and BaeSR two-component system, and the σ^E-mediated system [5]. σ^E, encoded by *rpoE*, is induced not only by heat or ethanol but also, and uniquely, by disruption of protein folding in the periplasm [6]. On activation, σ^E transcribes target genes including those encoding chaperones and proteases targeted to the cell envelope that will refold or degrade misfolded proteins [6]. In *E. coli*, σ^E activity is under the control of three genes, *rseABC* (for regulator of sigma E), which are encoded immediately downstream of the sigma factor [6,7]. This genetic organization (*rpoE rseA rseB rseC*) is conserved in the genome of other bacterial species such as *Pseudomonas aeruginosa*. In this bacterium, the organization is *algUmucABCD*. AlgU is the functional equivalent to σ^E in *E. coli* [8]. Besides contributing to the stress response, *algU* (σ^E) also controls alginate production by *P. aeruginosa*. The exopolysaccharide (EPS) alginate has traditionally been considered the major EPS of *P. aeruginosa* biofilms during cystic fibrosis pathogenesis [9]. One of the most clinically significant characteristics of biofilm communities is that they are more resistance to antibiotics and host immunity than are their free-living conditions [9].

Little is known about the adaptations of *B. pseudomallei* for survival outside and inside the human host. During infection, *B. pseudomallei* must survive major environmental changes such as the presence of reactive oxygen intermediates within phagocytic cells, changes in osmolarity and even antibiotic treatment. To characterize the cellular role of σ^E, we identified the genomic region that comprises the *rpoE* operon and investigated its importance in *B. pseudomallei* survival under stress conditions, and in biofilm formation. To accomplish this, an *rpoE* operon mutant was constructed by insertional mutagenesis in order to examine its response to osmotic stress and reactive oxygen intermediates, its ability to produce biofilms, and its survival within phagocytic cells.

2. Materials and methods

2.1. Strains, media and growth

B. pseudomallei K96243 (kindly provided by Prof. T. Dharakul) and *E. coli* S17-1*λpir* [10] were routinely maintained in Luria–Bertani (LB) agar or broth. Pseudomonas agar base supplemented with SR103 from Oxoid (UK) was used as a selective medium to inhibit growth of *E. coli* after conjugation. Antibiotics chloramphenicol (40 μg ml^{-1}), tetracycline (60 μg ml^{-1}) and ampicillin (100 μg ml^{-1}) were used as required. Where needed, M9 minimal medium (Gibco, BRL) was supplemented with 2 M NaCl. Murine macrophage (J774A.1) cell line was obtained from the American Type Culture Collection (ATCC, Manasssas, Va.). Unless indicated otherwise, cells were cultured in Dulbecco's modified Eagle's medium (Gibco Laboratory, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) at 37 °C under a 5% CO_2 atmosphere. Biofilm, motility and cell aggregation assays were carried out as previously described [11–13].

2.2. Construction of a B. pseudomallei rpoE operon mutant

An *rpoE* operon knockout mutant was constructed as previously described [13]. Briefly, a 270-bp internal fragment of the putative *rpoE* coding sequence (CDS) was PCR amplified from *B. pseudomallei* K96243 genomic DNA using primers ALG36 (5′ CTC CAA ATA CCA CCG CAA GAT 3′) and ALG37 (5′ TAT CCC TTA GTT GGT CCG 3′) corresponding to *B. pseudomallei rpoE* nucleotide positions 78–98 and 332–349, respectively. The 270-bp PCR product was cloned into the *Eco*RV restriction site of the pKNOCK-Cm vector [14] to create pPK-1. This construct was introduced from *E. coli* S17-1*λpir* [10] into *B. pseudomallei* K96243 by conjugation. An insertion mutant was selected on Pseudomonas agar supplemented with SR103 containing chloramphenicol. Southern blot analysis and PCR assay confirmed insertion of the *rpoE* suicide plasmid at the correct location (data not shown).

2.3. RNA extraction and RT-PCR analysis

Extraction of total RNA by the modified hot acid phenol method was carried out as described previously [15]. For RT-PCR analysis, the *rpoE-bprE-rseB-mucD* operon was reverse transcribed into cDNA (Invitrogen, USA) then amplified with primers. The primers ALG58 (5′ GCG GAG ATG ATG GGT TGC C 3′) and ALG59 (5′ GCG GGG CTG GGC GGA CAA C 3′) were designed according to the *rpoE* and *rseB* genes of the *B. pseudomallei* sequences. The primers ALG70 (5′ TTG CGG CGG GCG TGG TCG T 3′) and ALG71 (5′ AGG GCT GGC AGA TCG CAC C 3′) were designed on the basis of the *rseB* and *mucD* genes of *B. pseudomallei*. PCR conditions were 35 cycles of 1-min denaturation at 95 °C, annealing at 59 °C for 50 s, and extension at 72 °C for 30 s.

2.4. Electron microscopy and stress response assays

To examine bacterial cell morphology by electron microscopy, *B. pseudomallei* was cultured on tryptic soy agar. After 48 h incubation, a small piece of agar with bacterial cells on the surface was removed, fixed