P1022

Paper Poster Session V

Assaying and explaining multidrug resistance in Gram-negative bacteria

Identification of *Klebsiella pneumoniae* genes involved in tigecycline-resistance using transposon mutagenesis

C. Fyfe[1], D. Norris[1], J. Sutcliffe[1], T. Grossman[1]

[1]*Tetraphase Pharmaceuticals, Watertown- MA, USA*

Objective: To explore intrinsic mechanisms conferring resistance to tetracycline-class antibiotics, we constructed a transposon library in *Klebsiella pneumoniae* and selected for tigecycline-resistant mutants *in vitro*.

Methods: A transposon library in *K. pneumoniae* was generated by mating *E.coli* containing the λpir-dependent R6K plasmid pTnModRKm' with a rifampin-resistant isolate of *K. pneumoniae*. *K. pneumoniae* transconjugants were selected simultaneously on kanamycin (50 µg/mL) and rifampin (50 µg/mL) and the resultant transconjugants were pooled and stored. For tigecycline-resistance selection the transposon mutant pool (~8 x 10^6 kanamycin-resistant *Tn* mutants) was plated on Mueller Hinton agar containing 2 µg/mL tigecycline, ~4-times the minimal inhibitory concentration (MIC) for the parental strain. A total of 129 transposon mutants were isolated and confirmed to grow on 2 µg/mL tigecycline, and screened on escalating concentrations of tigecycline up to 16 µg/mL. Fifteen mutants having tigecycline MIC values ≥8 µg/mL were selected for characterization. Mutant chromosomal DNA was digested and self-ligated to produce Kan-ori_{R6K} plasmids that could replicate in an *E.coli* λpir strain; recovered plasmids were sequenced using primers derived from mini-*Tn*5 sequence. Complementation studies were performed using an arabinose-inducible pBAD plasmid, engineered to be selectable by gentamicin, expressing *K. pneumoniae lon, ramR* or Escherichia coli *lacZ* gene as a control. Selected mutants and the parents were tested in an invasion assay with RAW264.7 macrophage cells. Macrophages were infected for 2 hours to allow phagocytosis, extracellular bacteria were washed away and cells were incubated with medium containing gentamicin to kill residual extracellular bacteria. Samples were taken at 2 and 24 hr post-infection to quantify intracellular colony forming units (CFUs).

Results: Thirteen of 15 insertions mapped to the *lon* gene encoding Lon protease, a central player in the bacterial stress responses in other organisms. Phenotypic characterizations showed that *lon* mutants had increased sensitivity to UV light, consistent with the role of Lon in the DNA damage response. Several recovered mutants harbored point mutations in the *ramR* gene, either alone or in conjunction with a *lon::Tn*5 insertion. Parental antibiotic susceptibility phenotypes were recovered in mutants carrying plasmids overexpressing *lon* or *ramR;* UV sensitivity was reversed by a plasmid expressing *lon. Lon* mutants had reduced virulence in RAW264.7 cells, with at least 10-fold lower CFUs accumulating during infection versus the parental strain, consistent with reported studies in other organisms.

Conclusions: The *lon* gene was identified as an important factor in the *in vitro* susceptibility to tigecycline. While Lon has been implicated in the degradation of RamA, a positive regulator of multidrug resistance in *K. pneumoniae*, *lon* mutants have not yet been reported in isolates derived from clinical infections. Decreased virulence of *K. pneumoniae lon::*Tn mutants in RAW264.7 cells suggests *lon* mutants may not be viable, or difficult to recover, from *in vivo* infections.