Integrative study of *H.infuenzae*- Host interactions (Project II)

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Since the submission of the P01 application, we have made progress in a several areas relevant to the proposed studies. Some of this work was recently published **(Akerley and Lampe, 2002, see also A5.pdf; Bergman and Akerley, 2003, see A6.pdf)** and other work described in this report has been submitted for publication **(Wong and Akerley, subm.)**. Both of these papers are directly relevant to the proposal as described in this update. In addition, we have contributed experimental data (described below) to a collaborative manuscript being prepared with the members of HIC that integrates proteomics, metabolic modeling, and physiological information from mutational studies **(Kolker et al., to be subm.)**.

A. Comparative Genomics

In order to fully understand *H. influenzae*-host interactions it will be informative to relate results obtained with model strains such as Rd-b+ with clinical isolates. The type b (Hib) strain Eagan serves as a prototypical virulent strain capable of generating infections in animal models and it retains numerous virulence-associated loci absent in the Rd-b+ strain. We have initiated a genomic analysis of this strain to identify previously unknown 'Genetic Islands' present in Hib but absent in the Rd genome sequence. Preliminary data from this study was described in the proposal and now a complete description with additional data is available (Bergman and Akerley, 2003). This information will provide valuable insights into the full set of genes likely to be important for infection. While detailed characterization of such novel loci lies outside the scope of the current application, results of such studies will be complementary to the those obtained by HIC with Rd-b+. We also expect that the our genetic map of regions that differ from the currently sequenced Rd KW20 strain will facilitate efforts to obtain a high-quality genome sequence for the Eagan strain, a goal that could potentially be pursued by this consortium in collaboration with others in the field.

B. Genetic Systems

Efficient generation of conditional loss of function mutations provides an important tool for functional studies and is particularly important for characterizing essential genes or genes that are required for normal cellular growth, viability, and accurate DNA replication. However, promoter systems with sufficiently tight regulation for analysis of essential genes by conditional expression have not been characterized in *H. influenzae*. In the proposal, we have described use of a D-xylose inducible system for conditional expression. We have recently demonstrated the utility of this system for verification of an essential role in growth for a putative periplasmic lipoprotein in both *H. influenzae* Rd and virulent type b strains.

A second approach that will also have general utility in our studies of *H. influenzae* was used to further characterize this gene. Natural transformation and an ordered mutant collection spanning the *H. influenzae* genome provide the means to target any gene of interest for mutagenesis and temperature sensitive (TS) mutant isolation (see Fig. 1). This technique was applied to generate a conditionally lethal allele of the lipoprotein gene. The resulting TS mutation was directly mapped to a single amino acid substitution within a motif conserved in all 17 putative orthologs of this protein in diverse species and the mutation was shown to be sufficient to confer the TS phenotype.

These results provide a set of versatile genetic approaches for functional analysis of *H. influenzae* genes (Wong and Akerley, *submitted*). Our ability to generate D-xylose dependent strains by conditional expression of a given essential gene of unknown function suggests that this system will be applicable to other genes of interest. For example, one potential class of Fur regulated genes we expect

to identify in the proposed studies consists of genes required for growth or viability of *H. influenzae* (proposal section **D.3.c**) and the inducible system will be useful for analysis of such genes (see Fig. 1).

C. Integrative Studies

Taking advantage of the mutants generated by genome-scale mutagenesis of *H. influenzae* in our previous studies, we have begun to incorporate physiological experiments with proteomic and metabolic modeling being performed by HIC. This section presents initial data from one such project in which proteome data is used to identify the components of major metabolic pathways present in *H. influenzae* that change in response to redox conditions of growth. Modeling is then used to predict the relative influence of these pathways on *H. influenzae*'s physiology. The Akerley lab is participating in these efforts by examining phenotypes associated with mutations in relevant pathways (as described below). This data can provide specific confirmation of more global predictions made by modeling efforts. In the example shown here, proteomic data had indicated expression of fumarate reductase under both anaerobic and microaerophilic conditions (Kolker et al., *to be subm.*). Our data confirm and extend this result by indicating that fumarate reductase is required for growth not only under low oxygen conditions, but also under aerobic conditions.

The observation that proteomics study detected fumarate reductase peptides in cultures grown both anaerobically and microaerophilically and the absence of a putative succinate dehydrogenase in *H. influenzae* suggested that this arm of the citric acid cycle operates differently in *H. influenzae* compared with other model organisms. To further investigate this hypothesis, an insertion mutation that disrupts the *H. influenzae frdA* gene (HI0835) predicted to encode fumarate reductase was isolated from an ordered collection of *mariner* transposon mutants generated previously and mapped by PCR (Akerley et al., 2002). Growth of the *frdA* mutant versus wild-type was compared under conditions of low and high oxygen supply (Fig. 2). Suprisingly, the mutant was attenuated for growth under both conditions indicating a requirement for fumarate reductase both aerobically and microaerophilically. The growth defect could not be reversed by addition of 10 mM succinate and 10 mM nitrate to the media. It is possible that fumarate serves as a primary electron acceptor under both conditions and that its role in conversion of fumarate to succinate does not account for the growth defect of the *frdA* mutant. In summary, these results demonstrate a role in growth for *frdA* under diverse redox conditions consistent with the mass spectrometry data.

Note in Clarification

In Aim 2 (D.2.a) we discuss analysis of potential post-transcriptional control by Fur. We recognize that a comprehensive analysis of this question is beyond the scope of the current proposal, but with application of RNA and protein expression profiling tools, we expect to gain an initial overview of Fur regulation in *H. influenzae* that will be useful for more targeted studies. For example, the proposed experiments will not directly address whether stability of specific transcripts is altered by Fur mediated control over a RhyB-like mRNA, implicated in mRNA degradation in *E. coli*, since neither method measures RNA stability. However, by examining regulatory targets both at the protein level and the RNA level, we are more likely to detect the full set of regulated targets. Since DNA microarrays measure total transcript levels without regard to whether it is fully intact, post-transcriptional message degradation may not result in a marked decrease in observed transcript levels. In cases in which protein levels are affected but array data does not indicate a change, subsequent studies are warranted to investigate whether a post-transcriptional affect could be occurring by quantitative time-course mRNA studies with a transcription inhibitor (e.g. rifampicin).

References:

Akerley, B. J., E. J. Rubin, V. L. Novick, K. Amaya, N. Judson, and J. J. Mekalanos. 2002. A genome-scale analysis for identification of genes required for growth or survival of *Haemophilus influenzae*. *Proc. Natl. Acad. Sci. USA*, 99, 966-971.

Akerley, B. J., and Lampe, D. J. 2002. Analysis of gene function in bacterial pathogens by GAMBIT. *In* V. L. Clark and P. M. Bavoil (eds.) Methods in bacterial pathogenesis. Part C. *Methods in Enzymology,* 358, 100-108.

Bergman, N. H. and B. J. Akerley. 2003. Position-Based Scanning for Comparative Genomics and Identification of Genetic Islands in *Haemophilus influenzae* Type b. *Infect. Immun.* 71:1098-1108.

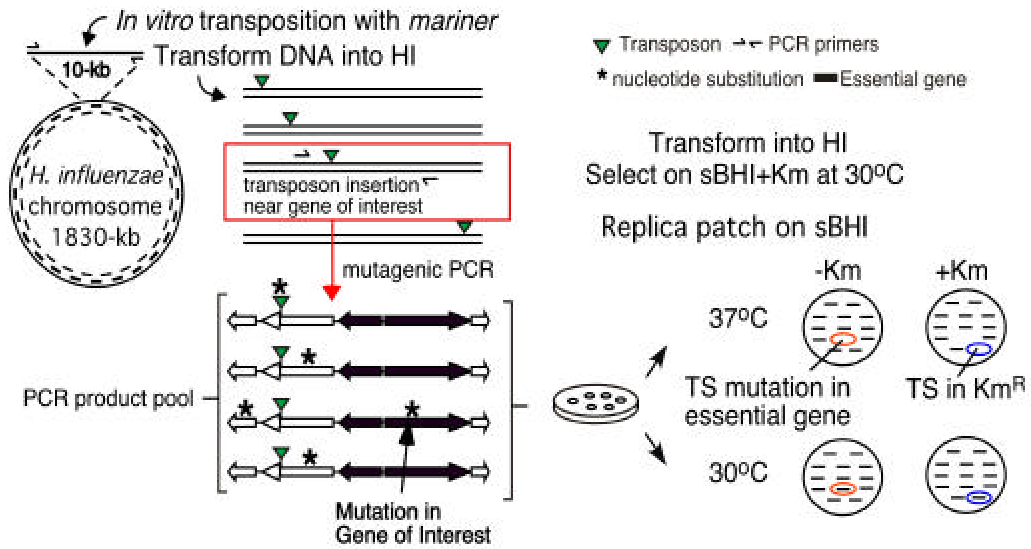
Wong, S. M., and B. J. Akerley. A Tightly-Regulated Conditional Expression System and Marker-Linked Mutagenesis Approach for Functional Genomics of *Haemophilus influenzae,* subm.

Kolker, E., Price, N., Raghunathan, A., Galperin, M. Y., Makarova, K. S., Xie, T., Purvine, S., Picone, A. F., Cherny, T., Tyler, R., Akerley, B. J., Munson, R. S. Jr., Smith, A. L., and Palsson, B. O. *In silico* metabolic model and protein expression of *Haemophilus influenzae* Rd strain KW20 in rich medium, to be subm.

Figure Legends:

Fig. 1 (next page, top). **D-xylose inducible system for conditional expression.** For details see section B.

Fig. 2 (next page, bottom). **The *frdA* mutant and parental wild-type Rd strains** were inoculated from overnight cultures to a starting OD600 of 0.003 and compared for growth in rich medium under low aeration (100 ml cultures in 125 ml shake flasks at 220 rpm.) versus high aeration (15 ml cultures in 125 ml shake flasks at 220 rpm). The 15 ml cultures were compared both in the absence and presence (suppl.) of 10 mM succinate and10 mM $NaNO_3$. Each point represents the mean of three experiments and standard deviations were below 10% of the means for all determinations.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10-kb
Transform DNA into HI
H. influenzae chromosome 1830-kb
transposon insertion near gene of interest
mutagenic PCR
PCR product pool
Mutation in Gene of Interest
Transposon
PCR primers
nucleotide substitution
Essential gene
Transform into HI
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-Km
+Km
37oC
30oC
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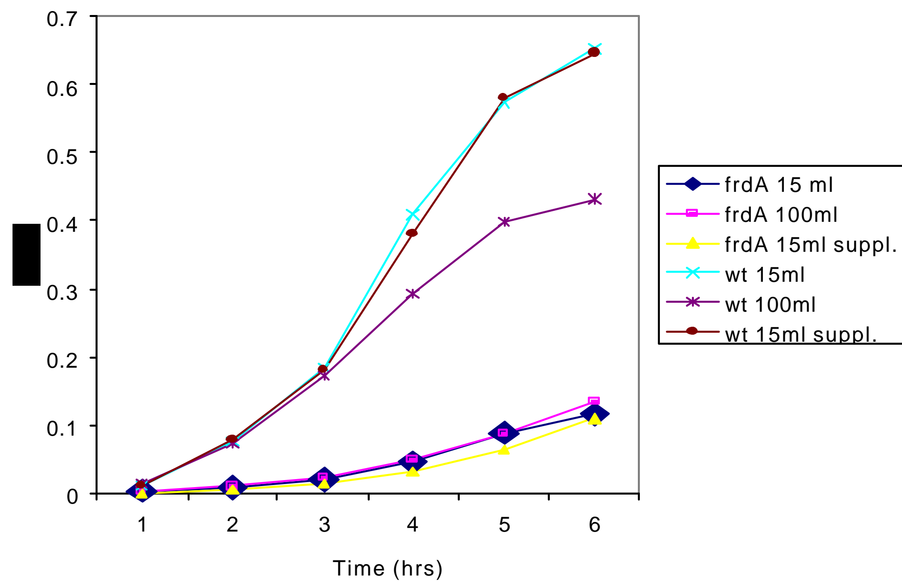
TS in KmR


Time (hrs)
frdA 15 ml
frdA 100ml
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