PROFILING *PSEUDOMONAS AERUGINOSA* WOUND INFECTIONS WITH HIGH-THROUGHPUT GENOMIC METHODS

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Abstract

Soft tissue infections caused by the opportunistic pathogen *Pseudomonas aeruginosa* are dangerous, difficult to treat, and have a significant economic impact on health care. In burn wound infections, *P. aeruginosa* grows and multiplies rapidly and often transitions into the bloodstream to cause systemic infections that can result in death. By contrast, in chronic wound infections, *P. aeruginosa* is highly persistent, resisting clearance by both physical and antimicrobial treatments, and a chronically-infected wound can persist for weeks or months. Thus, these two types of wounds represent highly different lifestyles for *P. aeruginosa*, yet many basic questions of bacterial physiology and metabolism during these infections remain. Here we address this gap by using high-throughput sequencing-based techniques to examine global gene expression and gene knockout fitness in mouse models of both burn and chronic wound infections. Specifically, we use RNA-Seq to characterize differential expression of both coding and noncoding RNAs *in vitro* and in model burn and chronic wound infections. We also subject a pool of ~100,000 PAO1 transposon mutants to growth in these same conditions, and profile mutant abundance by Tn-Seq. The results of these experiments reveal the crucial roles played by several primary metabolic pathways, motility, and many other phenotypes during wound infections. Finally, we compare the results obtained from applying these techniques to both chronic and acute wound infections to determine similarities and differences between these two distinct types of infections. We argue that, by simultaneously analyzing genome-wide gene expression and knockout fitness data in a variety of disease-related conditions, robust and powerful conclusions can be drawn about the lifestyles exhibited by pathogens in different infections

High-throughput genomics

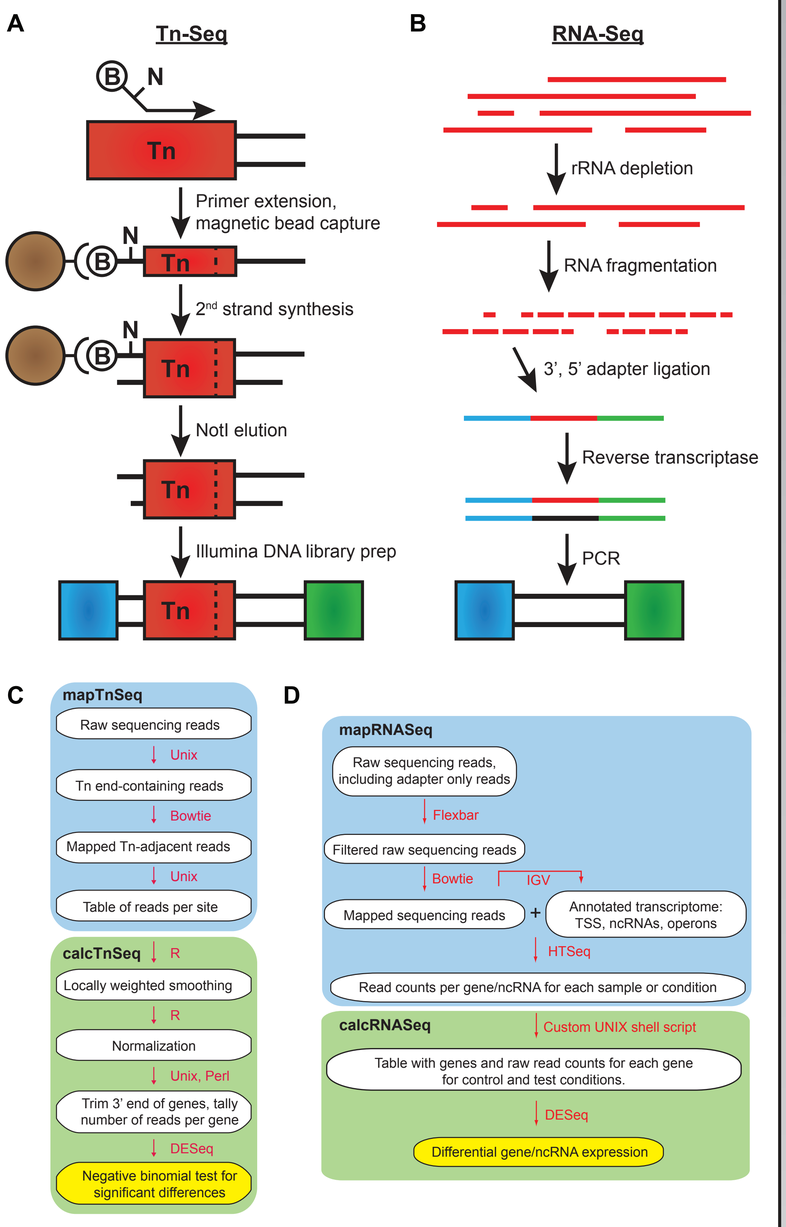

Figure 1. High-throughput genomic methods used in this study. (A) Tn-Seq methodology. Genomic DNA is sheared to approximately 400 bp and subjected to multiple cycles of primer extension using a biotinylated primer specific to the transposon end with a NotI site on the 5' end (the primer is designed so as to leave an 11 bp end for additional sequence quality control). Single-stranded primer extension products are bound to streptavidin-coupled paramagnetic beads, and a second strand is synthesized using random primers and Klenow DNA Polymerase fragment. Double-stranded DNA is eluted from the beads with NotI digestion, and DNA is subjected to standard Illumina genomic DNA library prep and high-throughput sequencing. (B) RNA-Seq methodology. Total RNA is depleted of both mammalian and bacterial rRNA with commercially available capture kits. Remaining RNA is fragmented and subjected to the NEBNext Small RNA Library Prep Set for Illumina, which involves the ligation of oligos to the 5' and 3' ends of the RNA fragments, synthesis of cDNA and PCR amplification of final sequencing libraries. (C & D) Custom analysis pipelines were developed for (C) Tn-Seq and (D) RNA-Seq data (Jorth *et al.* (2013), *J Bacteriol*) and used to determine differential gene expression and knockout fitness.

Tn-Seq replicate	**1**	**2**	**1**	**2**	**1**	**2**		**1**	**2**
# reads	19m	19m	41m	17m	16m	17m		35m	30m
# with Tn end	5.4m	2.2m	4.5m	2.1m	400k	1.7m		90k	190k
# sites identified	*30k	91k	101k	96k	20k	57k		*18k	*25k
RNA-Seq replicate	**1**	**2**	**1**	**2**	**1**	**2**		**1**	**2**
# reads	33m	25m	N.D.	N.D.	24m	74m		74m	23m
# reads mapped	26m	18m			5m	11m		1.6m	740k
Non-rRNA /tRNA reads	9.1m	8.1m			1.8m	3.8m		630k	220k

Table 1. Results of high-throughput sequencing and processing and gross analysis of the sequences obtained. Shown are raw counts obtained by both Tn-Seq and RNA-Seq performed on four conditions. (LB, Luria-Bertani broth culture at 37°C with aeration; MOPS-Succ, MOPS minimal media + 20 mM Succinate broth culture at 37°C with aeration; Burn wound, murine burn infection harvested at 24h post-infection; Chronic wound, murine chronic wound infection harvested at 4d post-infection; *, not considered in this analysis pending additional sequencing; N.D., not done, in progress; m, millions; k, thousands).

Growth in a wound invokes a large genomic response

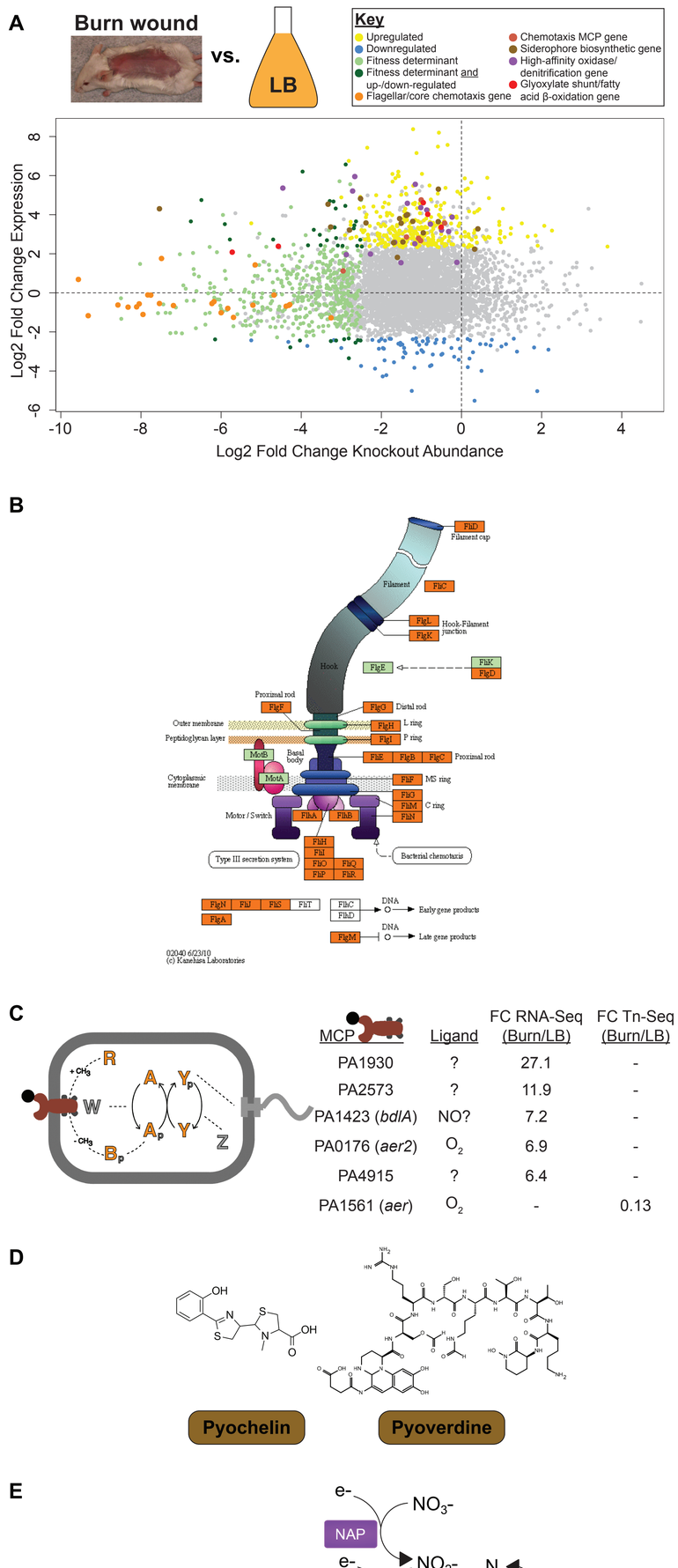

Figure 2. The genomic response of *P. aeruginosa* during burn wound infection. (A) Simultaneous plot of gene expression (RNA-Seq) and gene knockout fitness (Tn-Seq) data for every gene in the genome upon comparison of cells grown planktonically in LB broth to cells obtained from a monoculture murine burn wound infection (yellow & blue, genes significantly upregulated (yellow) or downregulated (blue) in burn wounds (fold change > 5, P < 0.01); light green, genes that significantly contribute to fitness in burn wounds (fold change > 5, P < 0.05); dark green, genes that both significantly contribute to fitness and are significantly up- or down-regulated in burn wounds; Other colors, see panels below). (B-F) Diagrams of specific systems found to be important in burn wound infection. Genes are colored according to their color in panel A. Systems identified include (B) the flagellum, (C) chemotaxis (MCP, methyl-accepting chemotaxis protein), (D) siderophores, (E) high-affinity terminal oxidases and denitrification enzymes, (F) the glyoxylate shunt and fatty acid β-oxidation. (G) Shown is a detail of Tn-Seq results obtained from comparing growth in MOPS minimal media with succinate as the carbon source and growth in the burn wound at the fatty acid β-oxidation genes *faoAB*. These results suggest that fatty acids are a major carbon source in burns.

Chemotaxis is a virulence factor in burn wounds

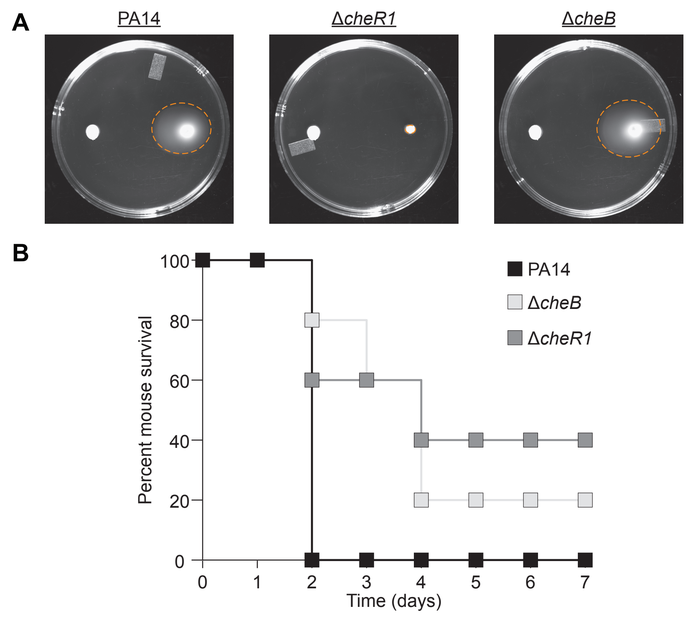

Figure 3. Core chemotaxis genes are required for virulence in burn wounds. (A) Phenotypes of wild-type (PA14) and chemotaxis gene transposon mutants (Δ*cheR1* and Δ*cheB*) grown on soft minimal agar with succinate as a sole carbon source (inoculated on right side of plates). A disc soaked in 20% casamino acids was placed on the left side of the plates to serve as a source of chemoattractant. (B) Survival assay of burned mice infected with the strains used above.

Certain biosynthetic pathways are dispensable in burns

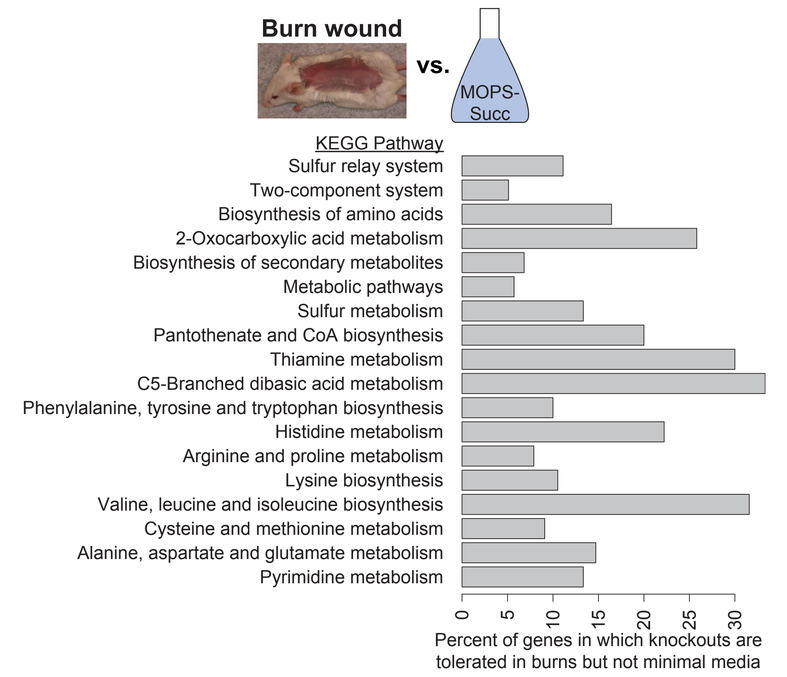

Figure 4. Biosynthetic pathways are significantly overrepresented among genes that contribute to fitness in minimal media but not in burn wounds. Shown is an enrichment analysis of genes that were identified as conditional fitness determinants in minimal media and not in burn wounds (Tn-Seq fold change > 2). The y axis contains KEGG pathways that were significantly enriched in this set (P < 0.05, Fisher's exact test) and the x axis displays the fraction of total genes in the genome belonging to each pathway that belong in this set. These data suggest that a number of biosynthetic intermediates may be utilized *in vivo*.

A second anaplerotic pathway is active specfically in chronic wounds

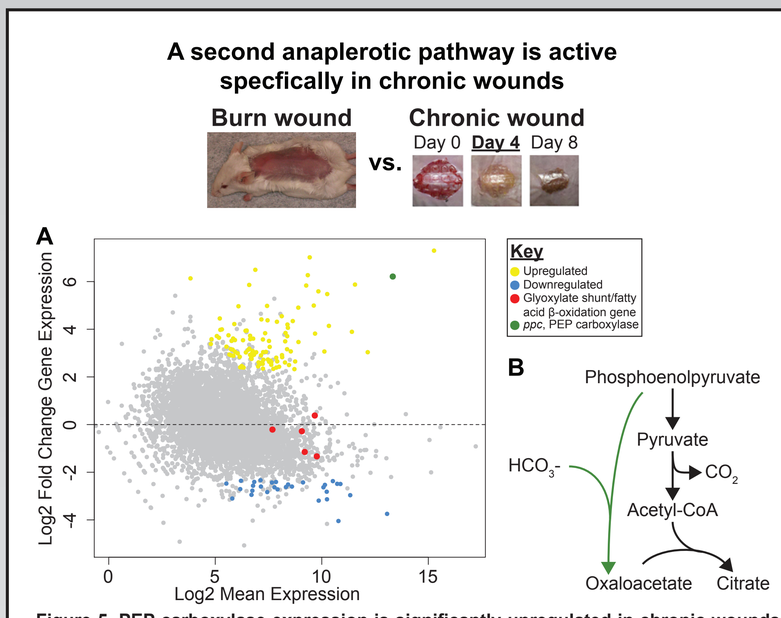

Figure 5. PEP carboxylase expression is significantly upregulated in chronic wounds as compared to burn wounds. (A) Shown is a plot of gene expression vs. fold change in gene expression (chronic wound/burn wound). Genes of the glyoxylate shunt found to be important in burn wounds (see Figure 2F) are highlighted in red, and *ppc*, which encodes PEP carboxylase, is highlighted in green. (B) Schematic showing the role of PEP carboxylase in replenishing the TCA cycle intermediate oxaloacetate. These data, taken with the identification of glyoxylate shunt genes as being important in burn wounds, suggest that anaplerosis is important in soft tissue infections by *P. aeruginosa*.

Acknowledgements and Contact

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