Differential Expression with DESeq2

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Why is differential abundance/expression/analysis?

One of the most common questions we ask as scientists is whether a variable of interest changes between two conditions. Perhaps we're measuring growth rate between wild-type and a mutant, or protein production in two types of media. Then, we need to assess statistical significance of this relationship: is our dependent variable **significantly** different between the two conditions?

If we're measuring a single dependent variable across two conditions, we can use common statistical tests like t-tests or Wilcoxon tests. However, our methods get more complicated if we want to measure the outcome of *multiple* variables from a single experiment. If there's only a few outcome variables, we could still do t-tests, and correct our p-values for multiple comparisons. We could also consider multivariate ANOVAs (MANOVAs). But what if we want to measure the outcome of **thousands** of variables? And what if these outcomes might be dependent on each other?

This statistical challenge (assessing the significance of a treatment on hundreds to thousands of outcomes) has become increasingly prevalent as 'omics methods have expanded. Metabarcoding, RNASeq, TN-Seq, and other sequencing technologies all allow us to simultaneously measure the levels of thousands of outcomes (species abundance, gene expression, strain enrichment) over two or more conditions. What's challenging is to then determine which outcomes are statistically significant.

There are many tools which have been developed to address these issues, and many papers have been published comparing different methods (e.g. this for RNA-Seq and this one for 16S). Different tools will have different levels of sensitivity (minimizing false negatives) and specificity (minimizing false positives) and no one tool is the "best" - I encourage you to read comparisons of tools and choose one that's best for your study design!

Today we'll be working with RNASeq data, and we'll use on of the most popular tools for gene expression analysis, `DESeq2`.

Our Data

We'll be working with previously published data looking at gene expression in two strains of *Staphylococcus aureus*. In [Waters et al., 2016](https://onlinelibrary.wiley.com/doi/full/10.1111/mmi.13404), they performed RNASeq, comparing gene expression in wild-type and a $\Delta codY$ mutant. CodY is a global transcriptional regulator which responds to depletions in branched-chain amino acids to modify gene expression. In the original paper, they tested a range of *codY* mutants across a spectrum of activity; today we'll just use one of them.

There's been a few steps which I've done, before we get started in this tutorial. We received sequencing data for two technical replicates of each condition. This data was cleaned and trimmed, and aligned to a reference genome (MRSA252). This produces .bam files. Then, I used the `featureCounts` command in the `subread` package to summarize reads for each feature (gene). We'll load and work with those featureCounts today to start the lesson.

```
# PREVIOUS WORK
bam_files <- list.files(path="data",
                        pattern="*.bam",
                        full.names=TRUE)

feature_counts <- Rsubread::featureCounts(files=bam_files,
                          annot.ext = "data/MRSA252_annotations.gff3",
```

```
                            isGTFAnnotationFile = T,
                            GTF.attrType = "ID",
                            GTF.featureType="gene",
                            GTF.attrType.extra = "Name")

save(feature_counts,
     file = "data/feature_counts.RData")
```

Loading packages and feature counts

First, we'll load necessary packages for today. Next, we'll load our featureCounts data, and explore a bit about the object.

```
library(tidyverse) # Necessary for data manipulation
library(DESeq2) # Tests differential gene expression
library(ggrepel) # For plotting non-overlapping text labels
library(ggmagnify) # Optional; to produce inset plot

load("data/feature_counts.RData") # Load feature counts

glimpse(feature_counts) # Look at list
```

```
List of 4
 $ counts    : int [1:2659, 1:4] 9849 11802 1144 7491 14959 27728 1903 244 7866 614 ...
  ..- attr(*, "dimnames")=List of 2
  .. ..$ : chr [1:2659] "gene-SAR0001" "gene-SAR0002" "gene-SAR0003" "gene-SAR0004" ...
  .. ..$ : chr [1:4] "codY_R61K_1.bam" "codY_R61K_2.bam" "wt_1.bam" "wt_2.bam"
 $ annotation:'data.frame': 2659 obs. of  7 variables:
  ..$ GeneID: chr [1:2659] "gene-SAR0001" "gene-SAR0002" "gene-SAR0003" "gene-SAR0004" ...
  ..$ Chr   : chr [1:2659] "BX571856.1" "BX571856.1" "BX571856.1" "BX571856.1" ...
```

```
  ..$ Start : chr [1:2659] "517" "2156" "3670" "3912" ...
  ..$ End   : chr [1:2659] "1878" "3289" "3915" "5024" ...
  ..$ Strand: chr [1:2659] "+" "+" "+" "+" ...
  ..$ Length: int [1:2659] 1362 1134 246 1113 1932 2661 831 1515 1287 696 ...
  ..$ Name  : chr [1:2659] "dnaA" "dnaN" "SAR0003" "recF" ...
 $ targets   : chr [1:4] "codY_R61K_1.bam" "codY_R61K_2.bam" "wt_1.bam" "wt_2.bam"
 $ stat      :'data.frame': 14 obs. of  5 variables:
  ..$ Status         : chr [1:14] "Assigned" "Unassigned_Unmapped" "Unassigned_Read_Type" "U
  ..$ codY_R61K_1.bam: int [1:14] 15274105 93722 0 0 0 0 0 0 0 0 ...
  ..$ codY_R61K_2.bam: int [1:14] 14431023 76346 0 0 0 0 0 0 0 0 ...
  ..$ wt_1.bam       : int [1:14] 18335478 128682 0 0 0 0 0 0 0 0 ...
  ..$ wt_2.bam       : int [1:14] 17360453 98704 0 0 0 0 0 0 0 0 ...
```

The feature counts output is a list with four slots. The `counts` slot is the most important to us, as it contains the number of reads mapped to each feature (gene) in each bam file (condition). Another useful slot is `stat`, which shows how many reads were mapped for each replicate - this could be important if on condition or replicate had a very different number of assigned reads!

Next, to run DESeq2, we'll need to extract the count data from our `feature_counts` object and create a `DESeqDataSet`. This will need a separate dataframe, which describes the metadata for each sequencing file. In this case, the strain. In other designs,this could be sample collection time or media type.

```
 # Just access the counts, and only keep geens with have at least 1000 reads
just_counts <- feature_counts$counts[rowSums(feature_counts$counts) > 1000,]

head(just_counts)
```

```
             codY_R61K_1.bam codY_R61K_2.bam wt_1.bam wt_2.bam
gene-SAR0001            9849            9508    11457    10491
```

```
gene-SAR0002          11802          11294    14292    13737
gene-SAR0003           1144           1014     1503     1336
gene-SAR0004           7491           7356     8520     8190
gene-SAR0005          14959          14443    17735    16690
gene-SAR0006          27728          24750    32210    30508
```

```
# Create a dataframe which describes metadata for each seq. file
strain_info <- data.frame(file = c("codY_R61K_1.bam",
                                   "codY_R61K_2.bam",
                                   "wt_1.bam",
                                   "wt_2.bam"),
                          strain = c("codY",
                                     "codY",
                                     "wt",
                                     "wt"))

# Construct DESeq Dataset
deseq_ds <- DESeqDataSetFromMatrix(countData = just_counts,
                                   colData = strain_info,
                                   design = ~strain)
# The ~strain shows we want to test across the strain variable
```

Next, we'll run DESeq2 to test for differential gene expression.

```
deseq_object <- DESeq(deseq_ds)

glimpse(deseq_object)
```

```
Formal class 'DESeqDataSet' [package "DESeq2"] with 8 slots
  ..@ design            :Class 'formula'  language ~strain
```

```
.. .. ..- attr(*, ".Environment")=<environment: R_GlobalEnv>
..@ dispersionFunction:function (q)
.. ..- attr(*, "coefficients")= Named num [1:2] 0.00177 3.6679
.. .. ..- attr(*, "names")= chr [1:2] "asymptDisp" "extraPois"
.. ..- attr(*, "fitType")= chr "parametric"
.. ..- attr(*, "varLogDispEsts")= num 1.09
.. ..- attr(*, "dispPriorVar")= num 0.521
..@ rowRanges         :Formal class 'CompressedGRangesList' [package "GenomicRanges"] with
..@ colData           :Formal class 'DFrame' [package "S4Vectors"] with 6 slots
..@ assays            :Formal class 'SimpleAssays' [package "SummarizedExperiment"] with 1
..@ NAMES             : NULL
..@ elementMetadata   :Formal class 'DFrame' [package "S4Vectors"] with 6 slots
..@ metadata          :List of 1
.. ..$ version:Classes 'package_version', 'numeric_version'  hidden list of 1
..$ betaPrior      : logi FALSE
..$ modelMatrixType: chr "standard"
..$ betaPriorVar   : num [1:2] 1e+06 1e+06
..$ modelMatrix    : num [1:4, 1:2] 1 1 1 1 0 0 1 1
.. ..- attr(*, "dimnames")=List of 2
.. ..- attr(*, "assign")= int [1:2] 0 1
.. ..- attr(*, "contrasts")=List of 1
..$ test           : chr "Wald"
..$ dispModelMatrix: num [1:4, 1:2] 1 1 1 1 0 0 1 1
.. ..- attr(*, "dimnames")=List of 2
.. ..- attr(*, "assign")= int [1:2] 0 1
.. ..- attr(*, "contrasts")=List of 1
```

The results of the `DESeq` command are a complex S4 object with lots of info about how the model was run. To access information that will be more relevant to us, we'll use a separate

command, `results`, and cleanup the object slightly.

```
deseq_results <- results(deseq_object) %>%
  as.data.frame() %>% # Convert to dataframe
  mutate(gene = row.names(.)) # Add new column of gene name


head(deseq_results)
```

```
             baseMean log2FoldChange      lfcSE       stat    pvalue      padj
gene-SAR0001 10296.422    -0.06003818 0.05879963 -1.0210639 0.3072242 0.5972292
gene-SAR0002 12710.686     0.03875767 0.05280028  0.7340429 0.4629226 0.7425669
gene-SAR0003  1237.270     0.15514202 0.10084554  1.5384123 0.1239478 0.3838753
gene-SAR0004  7871.376    -0.07031979 0.05700853 -1.2334960 0.2173908 0.5062019
gene-SAR0005 15891.432    -0.01337841 0.05279550 -0.2534005 0.7999587 0.9234335
gene-SAR0006 28646.292     0.01743278 0.06035557  0.2888346 0.7727079 0.9143417
                     gene
gene-SAR0001 gene-SAR0001
gene-SAR0002 gene-SAR0002
gene-SAR0003 gene-SAR0003
gene-SAR0004 gene-SAR0004
gene-SAR0005 gene-SAR0005
gene-SAR0006 gene-SAR0006
```

This gives us a data frame containing two very important pieces of information: `log2FoldChange` (how much that gene is enriched in one condition vs. the other) and `padj` (the adjusted p-value). We'll use this information to make our volcano plot. One annoying thing we might notice is that our genes are annotated by the GeneID - a unique identifier for each gene in this genome. However, we often want to the gene name itself. We'll access that info from out `feature_counts` object and join it to our DESeq results.

```r
# Pull out gene annotations
annotations <- feature_counts$annotation %>%
  select(GeneID, Name)

# Join gene names onto our results
clean_deseq_results <- deseq_results %>%
                            left_join(annotations,
                                      by = c("gene" = "GeneID")) %>%
  filter(Name != "codY") # Also, remove codY

head(clean_deseq_results)
```

```
  baseMean log2FoldChange      lfcSE       stat    pvalue      padj
1 10296.422    -0.06003818 0.05879963 -1.0210639 0.3072242 0.5972292
2 12710.686     0.03875767 0.05280028  0.7340429 0.4629226 0.7425669
3  1237.270     0.15514202 0.10084554  1.5384123 0.1239478 0.3838753
4  7871.376    -0.07031979 0.05700853 -1.2334960 0.2173908 0.5062019
5 15891.432    -0.01337841 0.05279550 -0.2534005 0.7999587 0.9234335
6 28646.292     0.01743278 0.06035557  0.2888346 0.7727079 0.9143417
          gene    Name
1 gene-SAR0001    dnaA
2 gene-SAR0002    dnaN
3 gene-SAR0003 SAR0003
4 gene-SAR0004    recF
5 gene-SAR0005    gyrB
6 gene-SAR0006    gyrA
```

Much better! While some genes aren't named, this is more informative than the Gene IDs.

Plotting our DESeq Results

There is no "one-good-way" to analyze differential abundance data. Popular choices include heat maps, volcano plots, and barbell plots, and large tables showing the biggest changes. As a primary researcher, you'll need to decide what data is interesting and worth showing. For today, we'll focus on a volcano plot, with the goal to see two things:

1. Were more genes turned "on" or "off" due to codY deletion?
2. What genes were **most** different between conditions?

There are thousands of genes in this dataset; it's impossible for us to show all of them. Often, we also make semi-arbitrary cut-offs as to what we deem "significantly different". One common decision are genes whose adjust p-values are < 0.05, and whose log2FoldChange is > 1 (so at least double). Below, we'll make a separate dataframe for these "strongly different" genes, which we'll use for labeling later on.

```
sig <- clean_deseq_results %>%
  filter(padj < 0.05, abs(log2FoldChange) > 1)
```

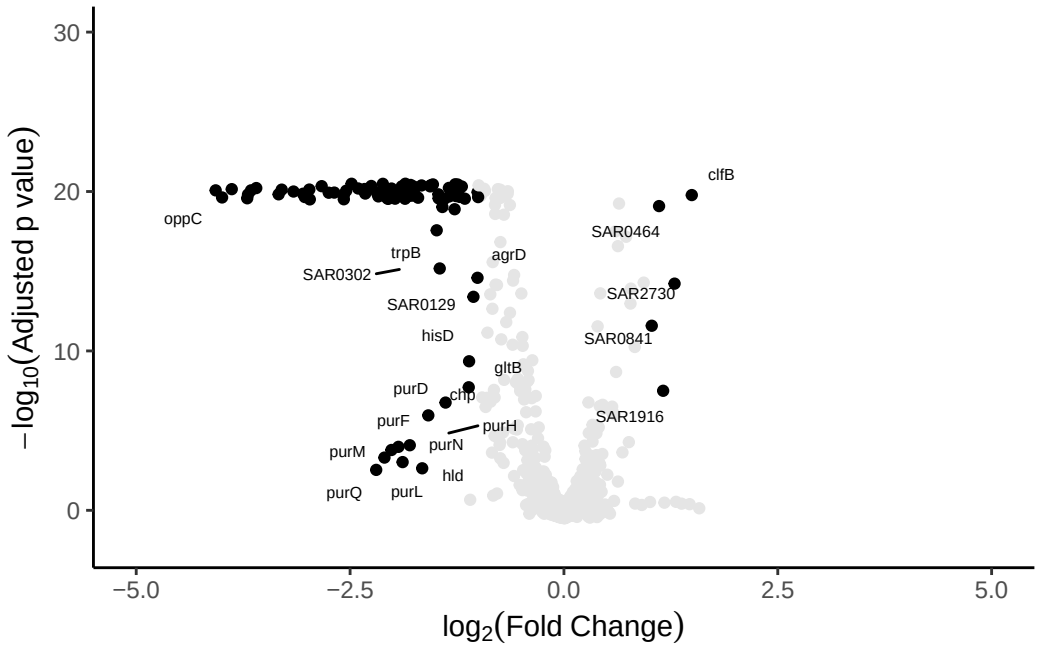
Making our Volcano Plot

You'll see there's a lot going on in the code below - that's okay! Good visuals take time and lots of small adjustments to have them look the best they can. During Hacky Hour, we'll build this plot up one step at a time, and explain my design choices for each layer.

```
start_plot <- ggplot(data = clean_deseq_results,
       aes(x = log2FoldChange,
                 y = -log10(padj+1e-20), # Add small value to limits y-axis
                 color = padj<0.05&abs(log2FoldChange)>1)) + # Color by significance and e
  geom_point(position = position_jitter(width = 0, height = .5)) +  # Add points with some
  coord_cartesian(xlim = c(-5, 5), # Make x-axis symmetrical
```

```
                  ylim = c(-2, 30)) +
  # Define colors for significant vs. insig. genes
  scale_color_manual(values = c("grey90", "black")) +
  # Make nice labels - bquote lets us include subscripts
  labs(x = bquote(log[2](Fold~Change)),
       y = bquote(-log[10](Adjusted~p~value))) +
  # Use a clean theme
  theme_classic() +
  # Remove legend
  theme(legend.position = "none") +
  # Add text labels for sig. genes - some tweaking here
  # to maximize the number and their location!
  ggrepel::geom_text_repel(data = sig,
                           position = position_jitter(width = 0, height = 0.5),
                           aes(label = Name),
                           point.padding = 2,
                           seed = 10,
                           size = 2,
                           max.overlaps = 12)

# Preview start_plot
start_plot
```

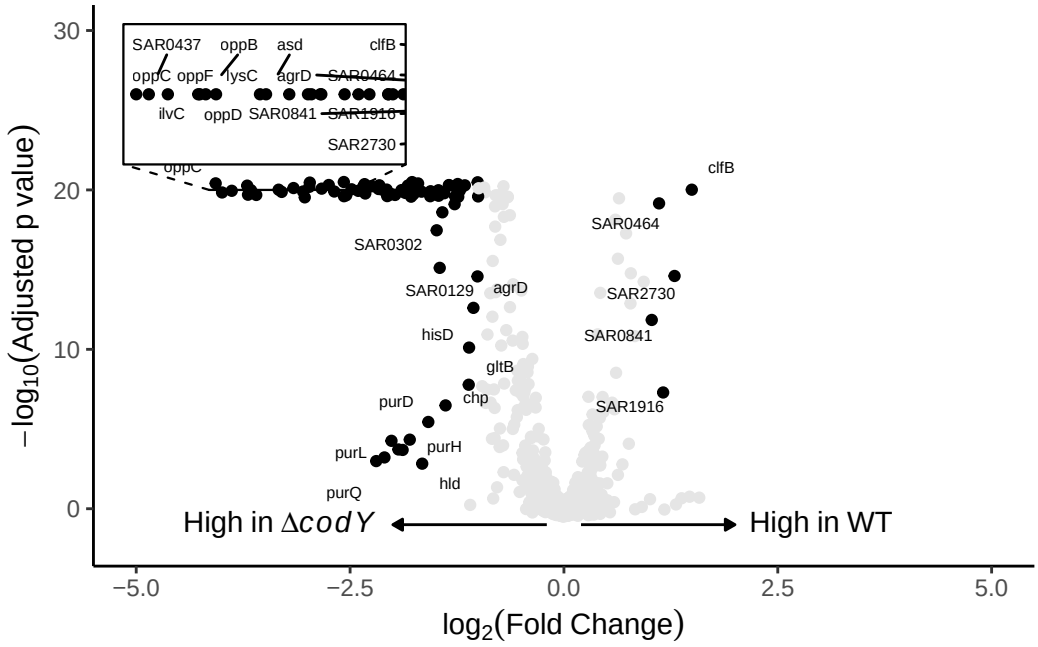



```
# Now, finish off with inset


final_plot <- start_plot +
  # Add inset
   ggmagnify::geom_magnify(aes(from = log2FoldChange < -2.5 & padj < 1e-20),
                           to = c(-5,-2,22,30)) +
  # Annotate arrows
   annotate(geom = "segment",
               x = c(-.2,.2),
               xend = c(-2,2),
               y = -1,
               yend = -1,
               arrow = arrow(type = "closed",
                             length = unit(0.02, "npc"))) +
  # Annotate arrow labels
```

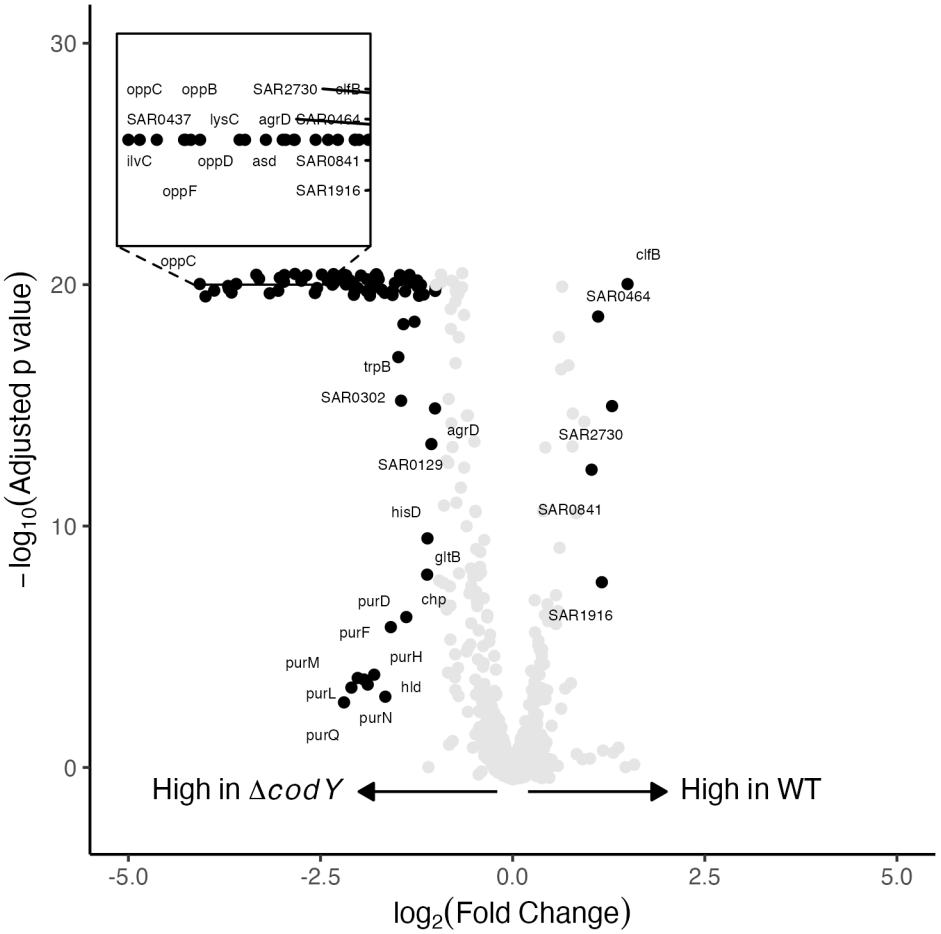
```
  annotate(geom = "text",
           x = c(-2,2),
           y = -1,
           label = c("High~'in'~Delta*italic(codY)",
                     "High~'in'~WT"),
           parse = TRUE,
           hjust = c(1.1,-.1))

# Preview final plot
final_plot
```




```
# Save final plot - note, all of our tweaking to get things just right
# had to be previewed in this final, saved plot!
ggsave(final_plot,
```

```
        filename  = "final_volcano.png",
        height = 5, width = 5, units = "in")
```



There we have it! I think that's quite nice. We see that more genes are expressed in tye codY-depleted strain, which makes sense as CodY is a repressor. We also find trends for which genes are derepressed, including purine synthesis (*pur* operon), the *opp* operon, which encodes an oligopeptide transporter, and genes for amino acid synthesis, including *lysC* and *ilvC*. All of these are important responses to nutrient starvation. We also see agrD pop up - an important two-component system which activates virulence responses, hinting towards CodY's important regulation of virulence. We've already learned a ton from just one plot that we made!