Investigating the Hypervirulence of a *Fusobacterium nucleatum* Ethanolamine Utilization Mutant in a Preterm Birth Model

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Abstract

Known to play a significant role in the development of oral biofilms, the oral pathobiont *Fusobacterium nucleatum* can spread to distal organs, including placenta and colon, promoting preterm birth and colorectal cancer. Since many bacterial pathogens acquire ethanolamine (EA) as a source of carbon and nitrogen, and EA is upregulated in the placenta during fetal development, we previously sought to examine the role of EA metabolism in fusobacterial virulence as this process is currently unknown in *F. nucleatum*. Because the EA-ammonia lyase EutBC catalyzes the breakdown of EA, we generated a *eutBC* mutant (Δ*eutBC*), initially predicting virulence attenuation of this mutant. Surprisingly, we found that Δ*eutBC* is hypervirulent, exhibiting its increased placental and fetal colonization and reducing pup survival. We now hypothesize that *eutBC* deficiency may cause intracellular accumulation of EA, which triggers upregulation of virulence factors that promote bacterial virulence and colonization. To investigate this, we began to isolate total RNA from the recovered placentas of animals infected with the parent or Δ*eutBC* strain for gene expression analysis of a few *Fusobacterium* predicted virulence factors by qRT-PCR. By creating a Python code to analyze gene expression data via a ΔΔCT method, we found that expression of genes coding for putative toxins and factors involved in oxidative stress are upregulated in Δ*eutBC*, relative to the parent strain. In our current experiments, we are employing RNA-seq to identify differentially regulated genes caused by *eutBC* deficiency at the genome-wide scale, aiming to reveal novel virulence factors contributing to the *F. nucleatum* pathophysiology.

Introduction

- *F. nucleatum* plays a key role in the development of oral biofilms and is linked to many human diseases including preterm birth. [1]
- Throughout pregnancy, ethanolamine presents in high concentrations in the placenta, helping maintain placental homeostasis and normal development of the fetus. [2]
- Bacteria utilize EA through a (eut) gene locus pathway that allows them to metabolize EA as a carbon source to begin pathogenesis. [3,4]
- *eutBC* encodes for the ethanolamine ammonia lyase which carries out the first major step in the breakdown of EA. [5]
- Previous studies in the lab found that deletion of *eutBC* led to a hypervirulent phenotype despite not being able to utilize EA as a nutrient source as shown below in *Fig* 1.

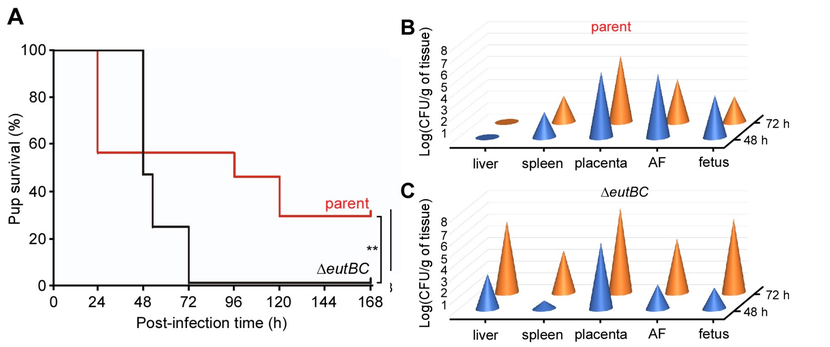

***Figure 1*- Hypervirulence of the *eutBC* mutant: (A)** A group of 5 pregnant mice was infected with ~ 5 x 10^7 CFU of the parent or Δ*eutBC* strain via the tail vein on day 16 or 17 of gestation, and pup survival was recorded. The statistical differences were analyzed by Mantel-Cox (** P<0.01).
(B-C) A similar procedure in A was performed, except that at indicated time intervals after injection, different organs and amniotic fluid (AF) were harvested for bacterial numeration.

Methods

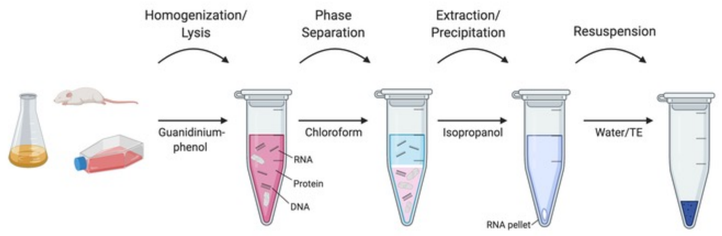

***Figure 2*- RNA Extraction:** A group of 4 pregnant mice was infected with ~ 5 x 10^7 CFU of the parent or Δ*eutBC* strain via the tail vein on day 16 or 17 of gestation. Placentas were collected at 48hr and 72hr post injection. Host and bacterial RNA were extracted using a TRIzol Bacterial Isolation kit. RNA was converted into cDNA and gene expression of known *F. nucleatum* virulence factors were measured via qRT-PCR.

```
main.py ×
119 def main_multi():
120     file_set = ('__') #change '__' to file name of choice in this format 'file_name.txt'
121     #separate by commas and make sure to include quotation marks in file name
122     num_samples = int(input('Enter number of samples per gene: '))
123     ref_gene = input('Enter name of reference gene: ')
124     file_name = input('Enter new file name: ') + '.csv'
125     for file in file_set:
126         Data = parse_data(file)
127         Averages = avg_cq(Data)
128         cq_per_gene = dict_sample_avg(Data, Averages, num_samples)
129         delta_dict = delta_cq(cq_per_gene, ref_gene)
130         ddct_dict = delta_delta_cq(delta_dict)
131         fold_2change = fold_change(ddct_dict)
132         all_calc = (cq_per_gene, delta_dict, ddct_dict, fold_2change)
133         dataframe_tofile(Data, all_calc, file_name)
134
135 main_multi()
136
```

A	B	C	D	E	F	G	H	I	J
Average 3x	hicA	hicB	radD	rs10740	rs00590	rs01010	rs07100	16s	1x
galk-48 (1)	34.2025027	35.1620891	30.5872376	33.9966647	32.8002996	30.8138908	31.8628416	21.8600246	
eutBC-72 (1)	29.2769056	29.5787975	26.5914955	27.9013976	27.4972292	27.0766037	27.8097023	17.6988023	
delta ct	hicA	hicB	radD	rs10740	rs00590	rs01010	rs07100	16s	
galk-48 (1)	12.3424781	13.3020645	8.72721301	12.1366401	10.940275	8.95386616	10.002817	0	
eutBC-72 (1)	11.5781033	11.8799952	8.89269317	10.2025953	9.79842689	9.37780144	10.1109	0	
ddct	hicA	hicB	radD	rs10740	rs00590	rs01010	rs07100	16s	
galk-48 (1)	0	0	0	0	0	0	0	0	
eutBC-72 (1)	-0.7643748	-1.4220692	0.16548016	-1.9340448	-1.1418481	0.42393528	0.10808303	0	
2^-ddct	hicA	hicB	radD	rs10740	rs00590	rs01010	rs07100	16s	
galk-48 (1)	1	1	1	1	1	1	1	1	
eutBC-72 (1)	1.69863378	2.67969583	0.89163171	3.82125036	2.20663516	0.74538863	0.92782008	1	
Average 3x	hicA	hicB	radD	rs10740	rs00590	rs01010	rs07100	16s	2x
galk-48 (1)	31.0069711	34.82718	30.7564365	32.4619075	35.2799353	30.4093835	32.4124378	21.7340697	
eutBC-72 (1)	26.43589	28.8359876	26.3616835	27.7310232	27.4281703	27.0614704	27.6328836	17.831365	
delta ct	hicA	hicB	radD	rs10740	rs00590	rs01010	rs07100	16s	
galk-48 (1)	9.27290138	13.0931103	9.02236678	10.7278379	13.5458656	8.67531379	10.6783681	0	
eutBC-72 (1)	8.60452497	11.0046226	8.53031853	9.89965816	9.59680524	9.23010536	9.80151863	0	
ddct	hicA	hicB	radD	rs10740	rs00590	rs01010	rs07100	16s	
galk-48 (1)	0	0	0	0	0	0	0	0	
eutBC-72 (1)	-0.6683764	-2.0884876	-0.4920483	-0.8281797	-3.9490603	0.55479156	-0.8768495	0	
2^-ddct	hicA	hicB	radD	rs10740	rs00590	rs01010	rs07100	16s	
galk-48 (1)	1	1	1	1	1	1	1	1	
eutBC-72 (1)	1.5892834	4.25301998	1.40644024	1.7754438	15.4449184	0.6807554	1.83636076	1	
Average 3x	hicA	hicB	radD	rs10740	rs00590	rs01010	rs07100	16s	3x
galk-48 (1)	30.6307975	36.2432194	33.3406864	36.2001393	36.7434084	32.0599841	32.8970744	22.9138901	
eutBC-72 (1)	26.970281	29.6756556	27.0324982	28.212347	29.8390151	27.9814369	28.1321157	18.4157978	

***Figure 3*- ΔΔCt Method through Python:** To measure gene expression from qRT-PCR, a delta delta Ct method is used from measured Cq values. To improve efficiency of these calculations, we created a code in python that returns our final 2^-ddCt calculation as well as other calculation used in the method in a csv. An example of the main function and the formatted file is shown above.

Results

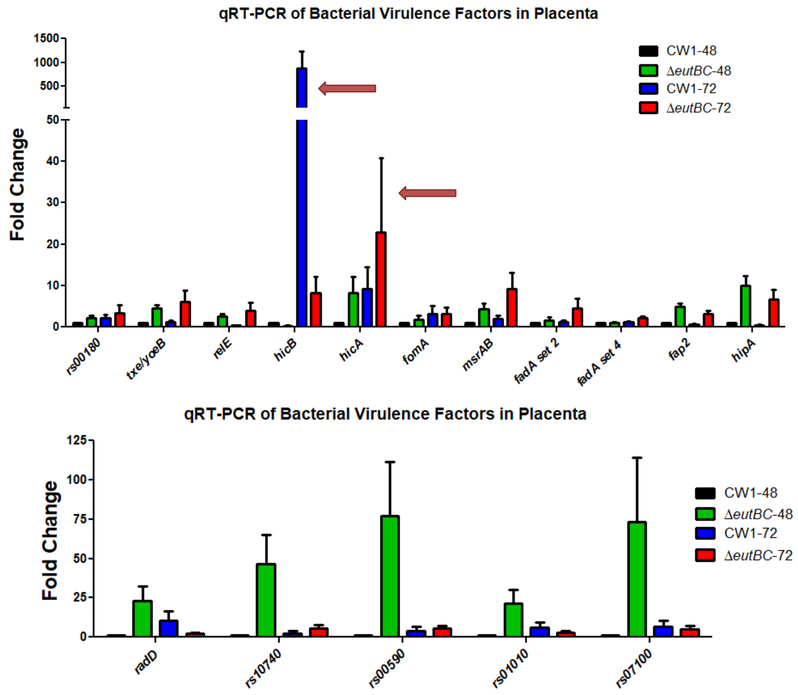

***Figure 4*- Comparison of virulence factors expression from recovered placentas:** After RNA extraction, qRT-PCR was performed for comparison of gene level expression between the parent strain at 48hrs to the other timepoints and mutant strain. Error bars indicate mean and standard deviation of three biological and technical replicates.

Conclusion

- Results from our python code do more efficiently calculate all needed values from the ΔΔCt method from multiple files/replicates at once.
- Our study shows that there are certain virulence factors being upregulated in a higher EA concentration environment, which can contribute to the hypervirulent phenotype of Δ*eutBC*.
- Our second graph shows that LPS genes expressed consistently in our mutant strain at 48 hours, but not at 72 hours.
- Our first graph highlights, *hicA* and *hicB*, proteins from a type II toxin/antitoxin that showed the greatest expression in our samples.
- The results support our hypothesis that increased expression of virulence factors in the Δ*eutBC* may contribute to its hypervirulent phenotypes.

Future Directions

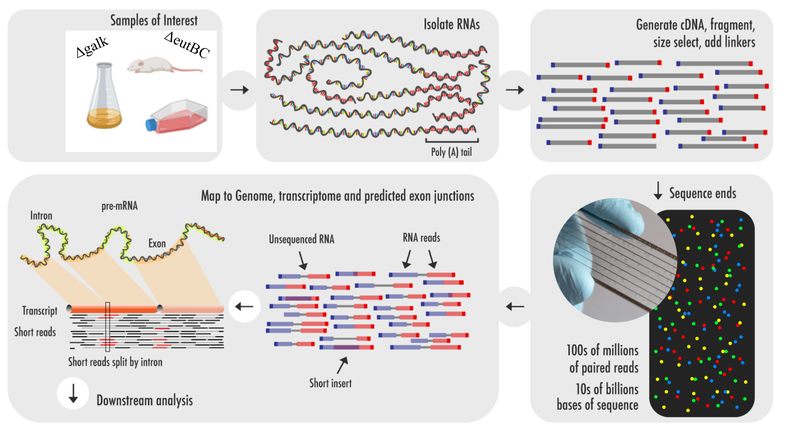

***Figure 5*- RNA-seq steps:** Repetition of injection and collection of placentas with our parent and mutant strains. RNA extraction in order to map our samples with transcriptome.

- While qRT-PCR gives us some insight on a few potential virulence factors expressed in the *eutBC* mutant, RNA-seq analysis will provide an unbiased view on all virulence factors contributing to the hypervirulence of Δ*eutBC* at the genome-wide level.

Acknowledgements

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References

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