Effects of gram-negative bacterial outer membrane permeabilization on *Serratia marcescens'* gene expression

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Abstract

Our laboratory has focused on understanding the effects of outer membrane permeabilizer compound 48/80 on the intrinsic resistance of gram-negative bacteria to hydrophobic antibacterial agents such as the biocide triclosan. Previously obtained RNAseq data were analyzed to identify gene expression changes potentiated by compound 48/80 in the opportunistic pathogen *Serratia marcescens*. These data indicated greatly upregulated expression of three genes, each involved in repair of outer membrane damage by other antimicrobial agents. The objective of the present study was to clarify the bacterial response to compound 48/80 treatment using RT-qPCR to follow gene expression, with the ultimate goal of establishing a proposed mechanism of action for compound 48/80-induced outer membrane permeability. Previous work indicated that *S. marcescens* is one of the few species of bacteria intrinsically resistant to triclosan, and that compound 48/80 induces transient sensitization to triclosan. The RNAseq analyses revealed a 50-fold increase in expression of *slyB*, *phoP*, and *phoQ* subsequent to compound 48/80 administration and qPCR primers were created in order to further investigate their regulation. The Bacterial and Viral Bioinformatics Resource Center (BV-BRC) analysis tools confirmed the upregulation of the aforementioned genes and provided Fragments Per Kilobase Million Mapped Reads (FPKM) to enable selection of an appropriate qPCR housekeeping gene, *yfiR*. RNA has been prepared from expression time-courses in preparation for the RT-qPCR process, in which qPCR primers for *slyB*, *phoQ*, *phoP* and *yfiR* will be used to measure expression changes observed over time.

Introduction

Using RNAseq data, it was demonstrated that treating *Serratia marcescens* with combinations of the biocide triclosan and outer membrane permeabilizer compound 48/80 yielded transcriptional changes in comparison to untreated controls (Katz Amburn unpublished work). Numerous genes were significantly up-regulated and down-regulated:

- Up-regulated → 161 for 30 min & 47 for 150 min
- Down-regulated → 176 for 30 min & 68 for 150 min

It was noted that there was a significant up-regulation of three genes, *slyB, phoP,* and *phoQ*, after 30 minutes but not after 150 minutes of compound 48/80 treatment. *phoP* and *phoQ* encode the two-component regulatory system that responds to environmental signals and modifies transcription accordingly for numerous genes including *slyB* (1; https://www.uniprot.org/uniprot/P23837). A housekeeping gene was identified and will be used as a control for future qPCR. The long-term goal of monitoring gene expression levels using qPCR is to better understand the bacterial cellular response to compound 48/80 over time.

Results

- The FastQC analysis indicated that the reads were, overall, of excellent quality. The analysis also confirmed results from an earlier analysis that the expression of these three genes displayed a 55-fold increase at 30 min post-compound 48/80 exposure.

Table 1. Expression levels of three genes of interest (FPKM)

	30 minutes average		150 minutes average	
	control	**experimental**	**control**	**experimental**
slyB	141.48775	7995.725	318.909	442.304
phoP	50.4596	3254.67	97.433325	199.98725
phoQ	29.912225	1799.22	57.614875	126.19375
yfiR (housekeeping)	43.07	52.03	62.25	65.99

- Analysis using BV-BRC annotation indicated up-regulation of some genes that were not noted previously.

Table 2. Examples of genes not noted previously for the 150 min time point.

Description	log2 fold change values
hypothetical protein (KFL05122.1)	2.68
probable exported protein YPO2521	2.63
hypothetical protein (KFL02057.1)	2.60
hypothetical protein (identified as a member of the PATRIC local protein family PLF_613_00003281)	2.16
YbdZ; enterobactin biosynthesis	2.95
fes; enterobactin esterase	2.95

Table 3. Examples of genes not noted previously for the 30 min time point.

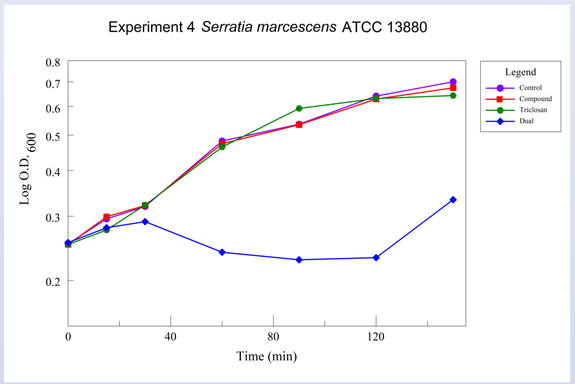
Description	log2 fold change values
pgaB; Biofilm PGA synthesis deacetylase PgaB	3.96
hypothetical protein (KFL03623.1)	3.23
hypothetical protein	2.63
ccmA; ABC-type efflux pump, duplicated ATPase component YbhF	2.59
FAD-dependent pyridine nucleotide-disulphide oxidoreductase	2.49
transcriptional regulator. LysR family	2.10
YebO; uncharacterized protein	2.06
Hypothetical, similar to sarcosine oxidase alpha subunit, 2Fe-2S domain	2.23
D-amino-acid oxidase (EC 1.4.3.3)	2.23
4-hydroxyproline epimerase (EC 5.1.1.8)	2.23

- qPCR primer design

Table 4. qPCR primer sequences designed for the genes of interest.

gene	primer ID	forward	reverse	primer pair info (F & R start)	product length
phoP	qphoPF; qphoPR	AGGTGGTGAGCAAAGATTCG	GTATTCCGCCTGCACTTTCT	500; 616	116
phoQ	Q PHOQ F; Q PHOQ R	CCGACGCTGGTCTTTATCTAC	CCTCCAGCCATTCCTTGTT	241; 341	100
slyB	q slyB F; q slyB R	TGATTGGCGGGCTGTTG	CCACTTCACCGATGCTGTTA	203; 314	111
yfiR	QyfiR F; QyfiR R	GTGGTGGTAGGCATCATCAG	GTATGCCGAAGGGCTGTT	181; 261	98

- Figure 2. Log OD of time course



Conclusions

- BV-BRC analysis results revealed 133 significantly up-regulated genes from 30 min post-compound 48/80 exposure, and 48 significantly up-regulated genes from the 150 min post-compound 48/80 exposure.
- BV-BRC annotation allowed identification of additional differentially expressed genes that were not noted in the earlier analysis.
- *yfiR* was identified as a housekeeping gene for future qPCR analysis of compound 48/80 treatment.
- Triplicate time courses for the four conditions (control, triclosan, compound 48/80, and dual treated) were run and samples at 7 time points were obtained for RNA isolation. Time points used were 0, 15, 30, 60, 90, 120, and 150 minutes.

Materials & Methods

- **BV-BRC Analysis**

Bacterial and Viral Bioinformatics Resource Center (BV-BRC) analysis tool allowed the re-analysis of the RNAseq results. The FastQC program was used to check quality of each read. Significantly induced and repressed genes were identified through differential expression analysis.

- **FPKM Calculation**

After BV-BRC mapped the reads to the *S. marcescens* genome, the expression level of each gene for each treatment was calculated as FPKM, Fragments Per Million Reads.

- **Housekeeping Gene Determination**

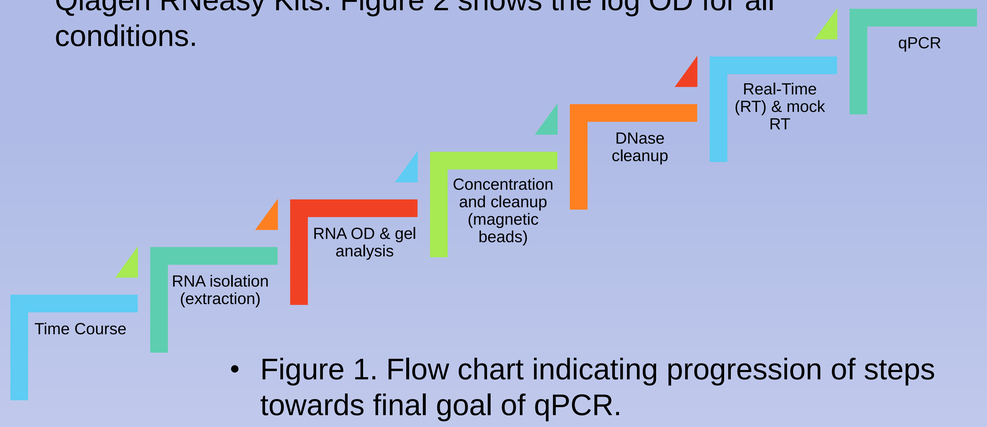
The genome was examined for genes whose FPKM values varied minimally among control and compound 48/80 treatments at the different time points. Genes with FPKM of lower than 10 cannot be tracked since RT-qPCR is less sensitive than RNAseq.

- **qPCR Primers**

PrimerQuest was utilized to design qPCR primers for the four genes, *slyB, phoP, phoQ*, and *yfiR***.** (https://www.idtdna.com/pages/tools/primerquest).

- **Experimental Time Course & RNA Extraction**

Grew *S. marcescens* and observed optical density at seven time points (0, 15, 30, 60, 90, 120, 150 m) under four conditions (control, triclosan, compound 48/80, and dual treatment). From these time points, RNA was extracted from samples using Qiagen RNeasy Kits. Figure 2 shows the log OD for all conditions.



- Figure 1. Flow chart indicating progression of steps towards final goal of qPCR.

References

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