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bio-protocol www.bio-protocol.org/e2891 Vol 8, Iss 12, Jun 20, 2018 DOI:10.21769/BioProtoc.2891

Characterizing the Transcriptional Effects of Endolysin Treatment on Established Biofilms of *Staphylococcus aureus*

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[Abstract] Biofilms are the most common lifestyle of bacteria in both natural and human environments. The organized structure of these multicellular communities generally protects bacterial cells from external challenges, thereby enhancing their ability to survive treatment with antibiotics or disinfectants. For this reason, the search for new antibiofilm strategies is an active field of study. In this context, bacteriophages (viruses that infect bacteria) and their derived proteins have been proposed as promising alternatives for eliminating biofilms. For instance, endolysins can degrade peptidoglycan and, ultimately, lyse the target bacterial cells. However, it is important to characterize the responses of bacterial cells exposed to these compounds in order to improve the design of phage-based antimicrobial strategies.

This protocol was developed to examine the transcriptional responses of *Staphylococcus aureus* biofilm cells exposed to endolysin treatment, as previously described in Fernández *et al.* (2017). However, it may be subsequently adapted to analyze the response of other microorganisms to different antimicrobials.

Keywords: Biofilms, Endolysins, *Staphylococcus aureus*, RNA-seq, Responses to antimicrobials

[Background] It is becoming increasingly clear that subinhibitory doses of antimicrobials may have a regulatory effect on different phenotypes of the target microbes, including biofilm formation, metabolism or virulence. Therefore, studying the potential impact of a novel compound on the target cells at low-level concentrations should be a part of the development process. Indeed, a very effective antibacterial agent that triggers production of virulence factors or antibiotic resistance determinants may not be a good candidate for therapeutic application. On the other hand, considering the physiological differences between biofilm and planktonic cells, it seems logical that the effect of new antibiofilm agents should be analyzed on biofilm-forming cells. Here, we describe a protocol for the analysis of transcriptional responses of biofilm cells upon exposure to subinhibitory concentrations of endolysins, phage-derived proteins that show great promise as biofilm removal agents. Thus, the transcriptome of endolysin-treated cells was compared to control cells by RNA-seq and differential expression of selected genes was later confirmed by RT-qPCR.

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Materials and Reagents

1. Standard Petri dishes (Labbox, catalog number: PDIP-09N-500)
2. Sterile 10 ml polystyrene culture tubes (Deltalab, catalog number: 300903)
3. Cuvettes for OD_{600} reading (Deltalab, catalog number: 303103)
4. 1.5 ml microcentrifuge tubes (SARSTEDT, catalog number: 72.690.001)
5. 12-well microtiter plates with Nunclon Delta surface (Thermo Fisher Scientific, Nunc, catalog number: 150628)
6. Sterile plastic loops (1 µl) (VWR, catalog number: 612-9351)
7. MicroAmp® Fast optical 96-well reaction plate with barcode (Thermo Fisher Scientific, Applied Biosystems, catalog number: 4346906)
8. MicroAmp® optical adhesive film (Thermo Fisher Scientific, Applied Biosystems, catalog number: 4311971)
9. Frozen stock of *Staphylococcus aureus* (for example, *S. aureus* IPLA1 from our laboratory collection) stored in glycerol at -80 °C
10. Filtered LysH5 endolysin stock stored in NaPi buffer with 30% glycerol at -80 °C (~350 µg/ml = 5.8 µM) purified as described previously (Gutiérrez *et al.*, 2014)
11. Agarose for electrophoresis (Conda, catalog number: 8008)
12. Glass beads, acid washed (≤ 106 µm, sterile) (Sigma-Aldrich, catalog number: G4649)
13. RNA protect® Bacteria Reagent (QIAGEN, catalog number: 76560)
14. Illustra™ RNAspin Mini Kit (GE Healthcare, catalog number: 25050071)
15. Chloroform (Merck, catalog number: 1024451000)
16. Ethanol (Fisher Scientific, catalog number: BP28184)
17. SUPERase-In™ RNase Inhibitor (Thermo Fisher Scientific, Invitrogen™, catalog number: AM2694)
18. Turbo DNA-free kit™ (Thermo Fisher Scientific, Invitrogen™, catalog number: AM1907)
19. DL-Dithiothreitol (Sigma-Aldrich, catalog number: D0632-5G)
20. Phenol, Molecular Biology Grade (Merck, Calbiochem, catalog number: 516724-100GM)
21. iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, catalog number: 1708841)
22. Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4367659)
23. Bacteriological agar (ROKO S.A.)
24. D(+)-Glucose (Merck, catalog number: 1.08337.1000)
25. Sodium chloride (NaCl) (Merck, catalog number: 1.06404.1000)
26. Potassium chloride (KCl) (VWR, BDH, catalog number: 437025H)
27. Sodium phosphate dibasic (Na_2HPO_4) (VWR, AnalaR NORMAPUR, catalog number: 102495D)
28. Potassium phosphate monobasic (KH_2PO_4) (Merck, catalog number: 1048731000)

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29. Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) (ITW Reagents Division, AppliChem, catalog number: 131965.1211)
30. UltraPure™ Tris Buffer (Thermo Fisher Scientific, catalog number: 15504020)
31. Glacial acetic acid (Merck, catalog number: 1.00063.2500)
32. 0.5 M EDTA (pH 8.0) (Alfa Aesar, USB, catalog number: J15701)
33. TSB medium (tryptic soy broth, Scharlab, catalog number: 02-200-500) (see Recipes)
34. TSA agar plates (see Recipes)
35. TSB medium supplemented with glucose (TSBG) (see Recipes)
36. Phosphate buffered saline (PBS) solution (see Recipes)
37. Sodium phosphate (NaPi) buffer (see Recipes)
38. Tris-acetate-EDTA (TAE) buffer (see Recipes)

Equipment

1. Pipettes (volume ranges: 1 µl-10 µl, 2 µl-20 µl, 20 µl-200 µl, 200 µl-1,000 µl)
2. Shaking (250 rpm) and static incubators at 25 °C and 37 °C
3. Spectrophotometer
   Note: It is used to measure optical density (OD_{600}) of cell culture.
4. Epoch microplate spectrophotometer (BioTek Instruments, model: Epoch)
5. Refrigerated centrifuge (Eppendorf, model: 5415 R)
6. FastPrep®-24 (MP Biomedicals, catalog number: 116004500)
7. Gel electrophoresis apparatus (Bio-Rad Laboratories, Mini-Sub® Cell GT Cell)
8. Vortex
9. 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Applied Biosystems, catalog number: 4351107)
10. Illumina HiSeq2000 platform
11. Computer equipped with four Intel Xeon E5-4650 v2 2.4GHz 25M 8GT/s 10-core processors, 256 GB RAM, and running CentOS Linux release 7.3.1611
    Note: The computer is for carrying out computation.

Software

1. FastQC (http://www.bioinformatics.babraham.ac.uk/projects/download.html#fastqc)
2. BowTie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) (Langmead and Salzberg, 2012)
3. EDGE-Pro (http://ccb.jhu.edu/software/EDGE-pro/) (Magoc *et al.*, 2013)
4. DEseq2 (http://bioconductor.org/packages/release/bioc/html/DESeq2.html) (Love *et al.*, 2014)

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Procedure

A. Biofilm formation and treatment (Figure 1)

1. Streak out *S. aureus* strain (IPLA1) from the frozen stock onto a TSB agar plate and incubate statically overnight at 37 °C.
2. To obtain three biological replicates, pick 3 isolated colonies of *S. aureus* from the agar plate with a sterile plastic loop and inoculate into three 10-ml polystyrene tubes containing 2 ml of TSB medium.
3. Grow bacterial cultures overnight at 37 °C, with shaking at 250 rpm.
4. Dilute the overnight cultures to an OD_{600} of 0.1 in TSBG medium (TSB supplemented with glucose), containing approximately 10^7 CFU/ml, and then make a 1:20 dilution in the same medium to prepare the inoculum for the biofilm assays.
5. Inoculate 2 ml from this cell suspension (approximately 5 x 10^5 CFU/ml) into each well of a 12-well microtiter plate (four wells per biological replicate).
6. Incubate the microtiter plate in static for 24 h at 25 °C.
   Note: In this case, the temperature used for biofilm formation and treatment was 25 °C, which represents treatment/disinfection at "room temperature". Nonetheless, the experiment could have also been performed at different temperatures; for instance, at 37 °C to represent treatment of human infection.
7. Remove the planktonic phase from the wells and wash the biofilms twice each with 2 ml of PBS.
8. For each replicate, add 1 ml of NaPi buffer alone to two wells and 1 ml of NaPi containing 10.94 µg/ml (0.18 µM) of LysH5 to the other two wells.
9. Incubate in static for 30 min at 25 °C.
10. Remove supernatant.
11. Wash twice with PBS.
12. Harvest cells corresponding to the same biological replicate and treatment in 1 ml of RNA protect® and 500 µl PBS by scraping with a pipette tip and transfer to a clean Eppendorf tube.
13. Process the samples according to the RNA protect® manufacturer's instructions.
14. Store at -80 °C or proceed to RNA purification.

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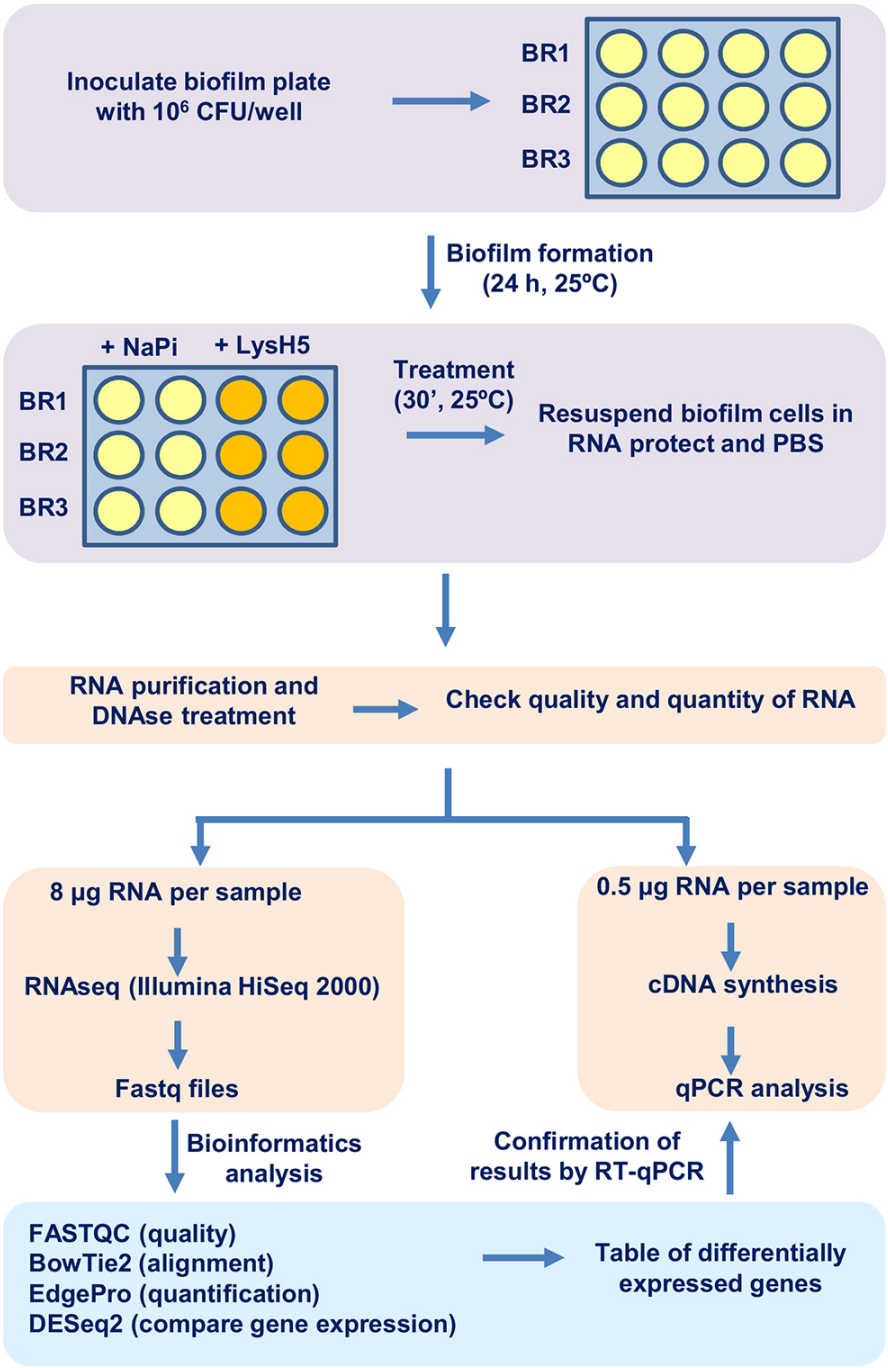

Figure 1. Schematic representation of the protocol. The different steps of this method include biofilm formation and treatment (purple), subsequent RNA purification and RNA-sequencing (pink) and, finally, computer analysis of the generated data (light green).

B. RNA purification and sequencing (Figure 1)

1. To achieve cell lysis, perform mechanical disruption of the cells with glass beads and phenol using FastPrep equipment.
2. After lysis, samples were centrifuged at 9,000 *x g* for 10 min at 4 °C.

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3. Transfer the upper phase to a clean tube and add 500 µl chloroform and then centrifuge for 5 min at 9,000 *x g* and 4 °C.
4. Transfer the upper phase to a clean tube and mix with 250 µl of ethanol by pipetting.
5. Transfer the samples mixed with ethanol to the columns provided with the illustra™ RNAspin Mini kit and perform the rest of RNA purification steps following the instructions provided by the manufacturer.
6. Elute in 50 µl of nuclease-free water and add 1 µl of SUPERase-In™.
7. Add 5 µl of Turbo DNase buffer and 1 µl of Turbo DNase per 50 µl sample and incubate for 30 min at 37 °C.
8. Add 1 µl of Turbo DNase per sample and incubate for another 30 min at 37 °C.
9. Remove DNase from sample with inactivation reagent as indicated by the manufacturer.
10. Add 1 µl of SUPERase inhibitor per 50 µl sample.
11. Check RNA quality and concentration by agarose gel electrophoresis (1% agarose) in TAE buffer and the Epoch microplate spectrophotometer (Figure 2). RNA concentrations obtained with this protocol usually range between 200 and 700 ng/µl.

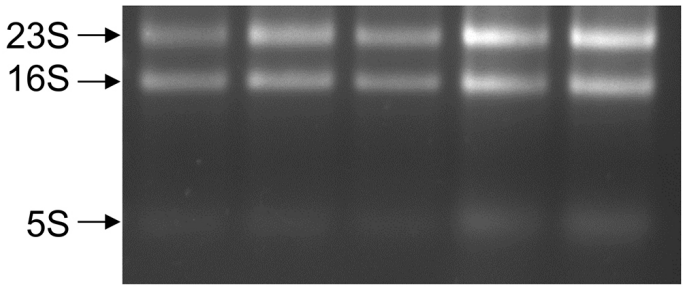

Figure 2. Agarose gel electrophoresis of total RNA from *S. aureus* biofilm samples. Aliquots (1-2 µl) from different RNA samples were run in a 1% agarose gel. Two bands corresponding to the 23S and 16S rRNAs should be visible and preferably in a proportion of 2:1 (23S:16S) indicating RNA integrity. Sometimes a lower band corresponding to 5S rRNA can also be observed.

12. Samples with A_{260}/A_{280} ratios ≥ 1.8 can be considered adequate for RNA-seq analysis. Otherwise, clean up the samples with the illustra™ RNAspin Mini kit following the protocol recommended by the manufacturer.
13. Take 8 µg of RNA from each sample and proceed with sequencing steps according to the protocols recommended by the manufacturer of the selected platform. For example, in this study samples were sent to an external service provider (Macrogen Inc., South Korea) for sequencing with an Illumina HiSeq2000 platform according to the protocols recommended by Illumina, generating 100-bp paired-end reads.

C. Computer analysis of the generated data (Figure 1)
   1. Check the quality of the reads in FASTQ format with FastQC.

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2. Download the reference genome in FASTA format (.fa or .fna), the protein table file (.ptt) and the RNA table (.rnt) from the NCBI archive (ftp://ftp.ncbi.nlm.nih.gov/genomes/archive/old_genbank/Bacteria/).
3. Run script "edge.pl" with arguments indicating the FASTQ files containing paired-end reads for each sample (-u and –v) as well as the three files mentioned above (-g, -p and –r) and the prefix for the output files names (-o). In a first step, EDGE-Pro will map the reads to the reference genome using program BowTie2 and create an alignment file as output (this file will be in sequence alignment map or SAM format). BowTie2 also indicates the percentage of alignment to the reference genome. Once completed this step, EDGE-Pro performs transcript quantification into Reads Per Kilobase of transcript per Million mapped reads (RPKMs). The output files containing the RPKM counts will end in ".rpkm_0".
   Example: /edge.pl -g SAreference.fna -p SAreference.ptt -r SAreference.rnt -u Lys_1-1.fastq -o Lys1 -v Lys_1-2.fastq
4. Run script "edgeToDeseq.perl" indicating the .rpkm_0 files to be analyzed in order to generate a table gathering the raw counts for each gene and each sample. This table will be saved in the output "deseqFile".
   Example: /edgeToDeseq.perl NaPi1.rpkm_0 NaPi2.rpkm_0 NaPi3.rpkm_0 Lys1.rpkm_0 Lys2.rpkm_0 Lys3.rpkm_0
   Note: This step is necessary because DESeq2 requires information on raw counts and not RPKMs.
5. Perform differential expression analysis between treated and untreated samples with DESeq2 by using the "deseqFile" from the previous step as an input. Select genes with an adjusted *P*-value < 0.05 for further analysis and save the table of differentially-expressed genes in .csv format.

D. Confirmation of RNA-seq results by RT-qPCR (Figure 1)
1. Convert 0.5 µg RNA from each sample into cDNA with iScript™ Reverse Transcription Supermix for RT-qPCR as indicated by the manufacturer.
2. Dilute cDNA samples 1:25 in nuclease-free water and use them as a template for qPCR.
3. To perform qPCR, add 2.5 µl aliquots from the different samples to each well of a MicroAmp® Fast optical 96-well reaction plate together with 3.25 µl of nuclease-free water, 0.25 µl of each primer from a 10 µM stock, and 6.25 µl of Power SYBR® Green PCR Master Mix.
4. Analyze each biological replicate in duplicate.
5. Determine changes in gene expression by using a reference gene (in this case *rplD*) according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), in which ΔCT = CT(target gene) - CT(reference gene) and ΔΔCT = ΔCT(target sample) - ΔCT(reference sample).

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Data analysis

For reproducibility, it is recommended to analyze three independent biological replicates (BR). Statistical analysis of RNAseq data was performed as part of the differential gene expression analysis with the DESeq2 package, and only those genes with adjusted *P*-values < 0.05 were selected for further analysis. Regarding fold-change, we normally set the cut-off at 2-fold change (log_2 fold-change = 1). However, in this case all genes displaying significant changes based on the adjusted *P*-values were analyzed further. The small changes are probably due to the fact that only part of the biofilm population was exposed to the antimicrobial. In addition to confirming the genes under the conditions described here, changes were further evaluated in a liquid culture exposed to endolysin LysH5. This analysis showed more evident changes in some of the genes identified by RNA-seq, which reinforced the idea that the transcriptional changes observed in the biofilm were indeed a result of endolysin exposure.

Recipes

1. Tryptic soy broth (TSB)
   30 g TSB medium
   Dissolve in 1 L ddH_2O and autoclave
2. TSA agar plates
   TSB medium with 2% agar
   Dissolve in ddH_2O and autoclave
3. TSBG medium
   TSB medium with 0.25% glucose
   Dissolve in ddH_2O and autoclave
4. Phosphate buffered saline (PBS) solution
   137 mM NaCl
   2.7 mM KCl
   10 mM Na_2HPO_4
   2 mM KH_2PO_4
   Adjust pH to 7.4
   Dissolve in ddH_2O and autoclave
5. NaPi buffer
   50 mM sodium phosphate
   Adjust pH to 7.4
   Dissolve in ddH_2O and autoclave
6. TAE buffer (50x stock solution)
   242 g of Tris
   57.1 ml of glacial acetic acid

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100 ml of 0.5 M EDTA (pH 8.0)
Add deionized water to 1 L

Acknowledgments

The development of this protocol was funded by grant AGL2012-40194-C02-01 (Ministry of Science and Innovation, Spain), AGL2015-65673-R (Program of Science, Technology and Innovation 2013-2017), Proyecto Intramural CSIC 201770E016, EU ANIWHA ERA-NET (BLAAT ID: 67), and GRUPIN14-139 (FEDER EU funds, Principado de Asturias, Spain). L.F. was awarded a Marie Curie Clarin-Cofund postdoctoral fellowship. P.G. and A.R. are members of the FWO Vlaanderen-funded PhageBiotics Research community (WO.016.14) and the bacteriophage network FAGOMA. This protocol was adapted from the previously published article Fernández *et al.* (2017).

Competing interests

The authors declare that they have no conflict of interest.

References

1. Fernández, L., González, S., Campelo, A. B., Martínez, B., Rodríguez, A. and García, P. (2017). Downregulation of autolysin-encoding genes by phage-derived lytic proteins inhibits biofilm formation in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 61(5).
2. Gutiérrez, D., Ruas-Madiedo, P., Martínez, B., Rodríguez, A. and García, P. (2014). Effective removal of staphylococcal biofilms by the endolysin LysH5. *PLoS One* 9(9): e107307.
3. Langmead, B. and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9(4): 357-359.
4. Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25(4): 402-408.
5. Love, M. I., Huber, W. and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15(12): 550.
6. Magoc, T., Wood, D. and Salzberg, S. L. (2013). EDGE-pro: Estimated degree of gene expression in prokaryotic genomes. *Evol Bioinform Online* 9: 127-136.

