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# Global gene expression analysis of *Streptococcus agalactiae* at the exponential growth phase

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**ABSTRACT**

A comparative overview of the global gene expression levels of S. agalactiae reference strain NEM316 at the exponential growth phase was done through RNA-sequencing. The expression levels of 47 genes potentially linked to virulence evidenced that: i) the major nuclease, GBS_RS03720/gbs0661, presented higher mean expression values than the remainder of DNase genes; ii) the genetic pilus island PI-2a genes presented higher mean expression values than PI-1 coding genes; and, iii) three virulence-associated genes ranked among the top-100 most expressed genes (GBS_RS07760, GBS_RS09445 and GBS_RS03485). Among this top-100, genes encoding proteins involved in "Translation, ribosomal structure and biogenesis" represented 46%. Curiously, genes with no assigned function were grouped in the category of highly expressed genes. As very little is known about the molecular mechanisms behind the release of DNases, preliminary assays were developed to understand whether direct DNA exposure would affect gene expression at the exponential growth phase. No differentially expressed genes were detected, indicating that follow-up studies are needed to disclose the complex molecular pathways (and stimuli) triggering the release of DNases. In general, our insights on the global expression levels of NEM316 at exponential growth phase with and without DNA exposure should open novel research lines to decipher S. agalactiae puzzling adaptation and virulence mechanisms, such as DNase production.

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## Introduction

Streptococcus agalactiae belongs to the family of streptococcaceae and is a common inhabitant of the healthy human gut and urogenital tract, particularly in women (1). As an opportunistic pathogen, S. agalactiae is a major cause of neonatal infections and mortality and has also emerged as a pathogen in elderly and immunocompromised adults (2). S. agalactiae is capable to adhere to various host cell types, namely epithelial cells of the vagina and the lung, endothelial cells and micro-vascular endothelial cells of the blood-brain barrier (3). However, the molecular mechanisms underlying the transition from colonization to infection have yet to be disclosed, and while the exact pathogenic features of S. agalactiae remain unrevealed, many virulence factors have been proposed to explain infection-related pathogenesis (4). In S. agalactiae reference strain NEM316 (genotype: III/ ST23) (5), isolated from an infected infant, several extracytoplasmic virulence factors have been identified, such as capsule, proteases, adhesins, haemolysin, pili and pigment. These factors mediate adhesion and epithelial cell invasion, and/or antagonize the immune system during phagocytosis (1, 6).

The understanding of the transcriptomic setting opens insights into the pathways of bacterial physiology, metabolism, and adaptation to changing environments (7, 8). Over the past decade, RNA sequencing (RNA-seq) has become indispensable for the transcriptome-wide analysis of differential gene expression. Together with improved computational tools for data analysis, innovations in RNA-seq technologies have been contributing to better-comprehending RNA biology and intermolecular interactions that govern RNA function, as well as transcriptional dynamics changes driving bacterial response and adaptation, to distinct growth conditions and external stimuli (9). Recently, RNA-seq technology has been successfully applied to clarify some molecular mechanisms of pathogenesis in S. agalactiae (8, 10-15).

The first comparative transcriptomic study in S. aga-

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lactiae evidenced several genetic factors likely important for the adaptation to its bovine host (in particular related to lactose metabolism) (10). In 2018, Hooven et al. (12) identified the gene products required for S. agalactiae survival in human whole blood, and Cook et al. (13) identified novel genes involved in the vaginal colonization by S. agalactiae, using a murine model. The contribution of CRISPR-associated protein-9 to S. agalactiae colonization and disease was also investigated by RNA-seq (14). While these targeted studies provided insightful data about specific S. agalactiae adaptive traits, the expression levels of this bacterium during its normal growth in the laboratory have not yet been systematized. In this study, we investigated the global gene expression, by RNA-seq, of S. agalactiae reference strain NEM316 during the exponential growth phase. As S. agalactiae evasion from the human defense mechanisms has been linked to the production of DNases (16, 17), we performed an additional preliminary assay whereby S. agalactiae reference strain NEM316 was exposed to DNA in order to evaluate its effect on gene expression.

## Materials and Methods

### Whole-genome sequencing of the laboratory reference strain NEM316

All experiments were conducted using the S. agalactiae reference strain NEM316 (ATCC 12403; genotype: III/ST23). The reference strain NEM316, maintained in the laboratory at -80ºC in cryopreservation tubes (Cryoinstant Red, VWR, Belgium), was cultured in Columbia agar supplemented with 5% sheep blood (Biomérieux, Marcy l'Etoile, France) at 5% CO2 for 24 h. These cultures were used to inoculate fresh Todd Hewitt broth supplemented with 0,5% yeast extract (THB) that were allowed to incubate without shaking at 37ºC with 5% CO2. Cell growth was monitored by measuring the optical density at 600 nm (OD600). At the middle exponential phase (OD600=0,2-0,5), 1 ml of bacterial cells were collected, centrifuged (3000 rpm, 10 min), resuspended in 200µl of PBS and immediately stored at -20ºC for further DNA extraction. Genomic DNA was extracted as previously referred (18), with minor changes. Briefly, bacterial cells were subjected to high-speed centrifugation (14,000 rpm) for 10 min at 4ºC. The pellet was digested for 2 h at 37ºC with 200 µl of Tris-EDTA buffer, pH 8.0, containing 10 U mutanolysin (Sigma-Aldrich, St. Louis, USA) and 15 mg/ml lysozyme (Sigma-Aldrich, St. Louis, USA) before treatment with 10 mg/ml proteinase K (Roche, Penzberg, Germany). Subsequently, DNA was extracted using the NucliSENS® EasyMag® (BioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The concentration of the extracted DNA was measured with QubitTM (ThermoFisher Scientific, Massachusetts, USA), and then subjected to Next Generation Sequencing in NextSeq 550 equipment (2x150bp) (Illumina, USA). The reads were deposited in the European Nucleotide Archive (ENA) (Bioproject PRJEB41294) under the accession number ERR4836035.

In order to evaluate the genetic differences between the genome of our laboratory passaged reference strain and the publicly available genome (GenBank accession number NC004368), two strategies were applied: i) de novo genome assembly using INNUca v.4.0.1 (https://github.com/B-UMMI/INNUca) (19), followed by genome alignment and inspection using MAUVE (http://darlinglab.org/mauve/mauve.html) (to inspect for the presence of structural changes, such as large indels); ii) reference-based mapping using Snippy v3.2 (to detect SNPs and small indels) (https://github.com/tseemann/snippy).

Clusters of Orthologous Groups (COGs) categories were assigned to the amino acid sequences retrieved from the NEM316 NCBI annotation (GenBank accession number NC004368) using the "cdd2cog" script (20) after RPS-BLAST+ (Reverse Position-Specific BLAST) (e-value cut-off of 1e-2), where only the best hit (lowest e-value) and first COG were considered.

### Bacterial culture for RNA-seq

Bacterial clones of NEM316 were grown in Columbia agar supplemented with 5% sheep blood (Biomérieux, Marcy l'Etoile, France), at 37ºC, 5% CO2 for 24 h and then inoculated in THB that were allowed to incubate without shaking at 37ºC, 5% CO2. Cell growth of S. agalactiae strain NEM316 was monitored by optical density at 600nm (OD600). At OD600=0,6 (exponential growth phase, see Supplementary material Figure S1) 1 ml of bacterial cells were collected, briefly centrifuged (3000 rpm, 10 min), resuspended in 1,8 ml of fresh THB and incubated for 0, 10 and 20 minutes at 37ºC in the presence of 200µl of PBS (used as control). For direct DNA exposure assays, the same procedure was performed, with the exception that 2µg/ml of DNA [human DNA from Hela cells – extracted using the DNA mini kit (Qiagen, California, USA) according to manufacturer's instructions] was added instead of PBS, and nuclease reaction was stopped by adding EDTA (0.5 M, pH 8.0) at 4ºC. For both conditions, 1 ml of each bacterial culture was collected and immediately subjected to high-speed centrifugation (14,000 rpm) for 10 min at 4ºC for RNA extraction. Note that we intentionally did not treat the bacterial culture from which RNA would be extracted with RNAprotectTM Bacteria Reagent (Qiagen, CA, USA) because a preliminary assay showed that this product degrades S. agalactiae RNA (data not shown).

### RNA extraction

RNA was extracted as previously referred (18), with minor changes, from bacterial cultures collected at 0, 10, 20 minutes with and without DNA exposure. Briefly, the cells were lysed in 200 µl of Tris-EDTA buffer, pH 8.0, containing 10 U mutanolysin (Sigma-Aldrich, St. Louis, USA) and 15 mg/ml lysozyme (Sigma-Aldrich, St. Louis, USA), at 37ºC during 90 min. The RNeasy mini kit (Qiagen, CA, USA) was used according to the manufacturer's instructions. Residual contaminant DNA was removed using 30 U RNase-free DNase (Qiagen CA, USA), and elution was performed with 40 µl of RNase-free water. RNA yield and purity were determined by absorbance measurement at 260 and 280 nm using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Extracted RNA from six bacterial cultures were finally stored at -80ºC until use.

### Bacterial mRNA preparation/purification

Bacterial mRNA was enriched using the Ribo-ZeroTM rRNA Removal Kit (Illumina, CA, USA) which removed abundant 16S and 23S rRNA from total RNA. The obtained bacterial mRNA was concentrated to a final volume of 14

µl using the RNeasy® MinElute® Cleanup Kit (Qiagen, CA, USA). The yield and integrity of the enriched mRNA samples were assessed with an Agilent Bioanalyzer, where the absence of rRNA readings is indicative of the success of rRNA depletion and purity of mRNA.

**RNA-seq**

Bacterial mRNA-enriched samples were subjected to library construction by a TruSeq Stranded mRNA sample preparation kit (Illumina, CA, USA). The obtained cDNA libraries were subjected to RNA-seq on a high-throughput MiSeq Illumina apparatus, targeting around 4M reads per 1Mbp. Sequence reads (2x75bp) were subjected to quality control and subsequently mapped to the S. agalactiae NEM316 genome (as obtained above) using Bowtie2 (21). Relative gene expression was quantified and normalized as fragments per kb of CDS per million mapped reads (FPKM) using the Cufflinks software (version 2.1.1; http://cufflinks.cbcb.umd.edu/). For comparative global gene expression analyses between normal growth conditions and the "direct DNA exposure", we applied HTSeq-count (https://htseq.readthedocs.io/en/release_0.11.1/count.html#) for read counting and state-of-the-art software for differential expression analysis (namely, EdgeR and Voom/Limma) using the interactive web-tool DEGUST (https://degust.erc.monash.edu/) (22).

The reads were deposited in ENA (Bioproject PRJEB41294) under the accession numbers ERR4836029, ERR4836030, ERR4836031, ERR4836032, ERR4836033, ERR4836034.

## Results and Discussion

**NEM316 whole-genome sequencing**

S. agalactiae reference strain NEM316 was isolated 18 years ago (5) and, since then, has been maintained in the laboratory. As such, in order to prepare RNA-seq assays, NEM316 was subjected to WGS to evaluate whether this laboratory passaged strain exhibited significant genetic changes in comparison with the publicly available genome (5) (GenBank accession number NC004368). Six genome-dispersed mutations were detected, including three small indels and three single nucleotide polymorphisms (SNPs), corresponding to three non-synonymous mutations (Table 1). Among these, we highlight an SNP in relA, which encodes an enzyme known to be involved in stringent response and bacterial adaptation to environmental stress (12). In S. agalactiae, relA knockout strains demonstrated decreased expression of β-hemolysin/cytolysin, an important cytotoxin implicated in facilitating invasion (12). Although the impact of these particular mutations at the transcriptomic level is unknown, we cannot rule out the possibility that they reflect either events of laboratory adaptation or errors in the first publicly released NEM316 genome sequence. Notwithstanding, we consider it good practice to analyze the genome backbone of strains subjected to gene expression (or other in vitro) assays, as a means to provide more complete data required to better interpret and discuss the results.

**NEM316 transcriptomic analyses**

The main goal of the present study was to evaluate the global gene expression dynamics of NEM316 S. agalactiae reference strain during the exponential phase using RNA-seq technology. This growth phase is particularly interesting when studying the transcriptional activity because most cells in the population are actively dividing, and this ensures that the expression of most of the S. agalactiae genes is assessed. As such, three-time points (0, 10 and 20 minutes) were evaluated during the exponential

**Table 1.** Genomic alterations for *S. agalactiae* NEM316 maintained in laboratory.

| Mutation location [a] | Type | Locus tag [b] | Old locus tag [b] | nt (aa) change [c] | Effect | Product |
|---|---|---|---|---|---|---|
| **1313729** | insertion | GBS_RS06735. pseudogene | gbs1273 | 811insA[d] | --- [d] | glucose-1-phosphate thymidylyltransferase |
| **1363940** | insertion | | | CTT > CTTT | | |
| **1741404** | deletion | | | GATATATA > GATATA | | |
| **1839016** | SNP | GBS_RS09285 | gbs1779 | A1077T (Glu359Asp) | Missense variant | Major facilitator superfamily transporter |
| **2001001** | SNP | GBS_RS10040 | gbs1928 | G1952C (Trp651Ser) | Missense variant | bifunctional (p)ppGpp synthetase/ guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase (relA) |
| **2129967** | SNP | GBS_RS10675 | gbs2055 | A11T (Lys4Met) | Missense variant | arginine repressor |

[a] Polymorphism location refers to the location in the reference genome: NEM316 (GenBank accession number NC004368). [b] Open Reading Frames (ORFs) designations according to reference genome: NEM316 (GenBank accession number NC004368). [c] The nucleotide changes in open reading frames are presented in the 5' to 3' direction. [d] This locus is a pseudogene in the reference genome (GenBank accession number NC004368) due to a one bp deletion as such NEM316 of our laboratory detains the original not truncated allele.

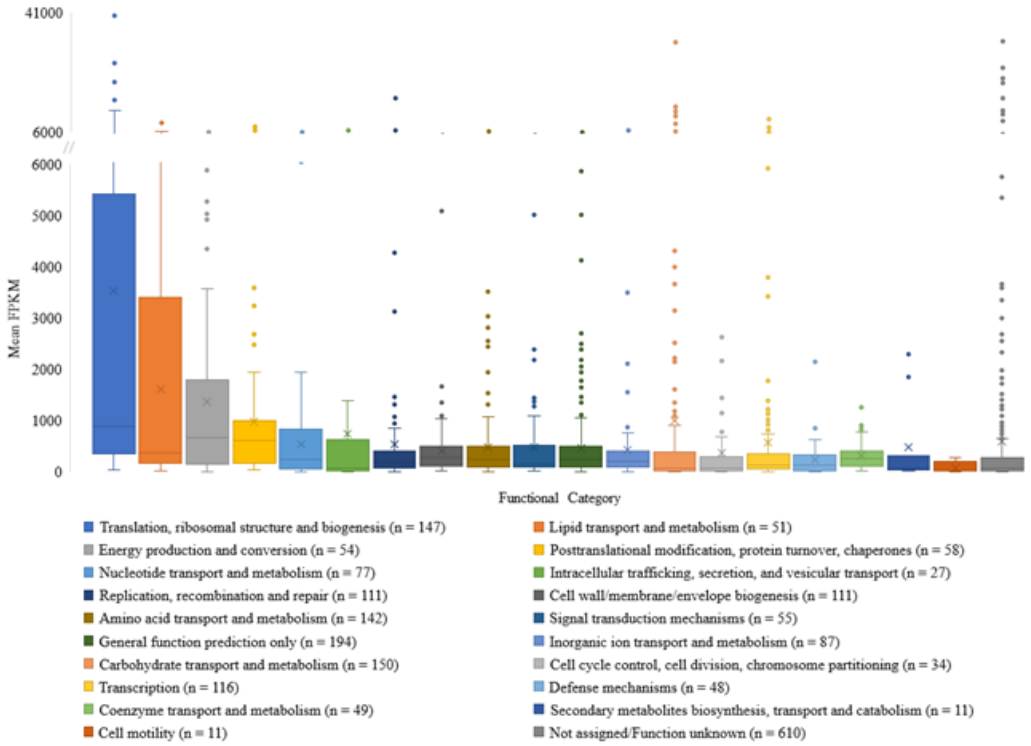


**Figure 1. Global gene expression by functional categories.** Box plot showing the distribution of gene expression levels by functional category, for reference strain NEM16 at the exponential growth phase. Values reflect the mean expression level evaluated at three-time points (0, 10 and 20 min) during exponential growth. Genes with mean expression levels < 1 FPKM were excluded from the analysis.

growth phase in THB.

Firstly, we ranked the S. agalactiae genes by expression level and correlated them with the gene functional category (Figure 1) (Supplementary material Table S1). Huge differences in the median expression levels were observed between different gene functional categories (Figure 1), with the top expressed functional category ("Translation, ribosomal structure and biogenesis") revealing a median expression value that was 55-fold higher than the less expressed functional category ("Cell motility"). The top three most expressed functional categories were "Translation, ribosomal structure and biogenesis", "Energy production and conversion" and "Posttranslational modification, protein turnover, chaperones". This result may not be surprising as during the exponential growth phase, the cell division rate is maximum and this implicates a high demand for proteins playing a role in translation and metabolism. The three functional categories with the lowest median expression levels were "Cell motility", "Intracellular trafficking, secretion, and vesicular transport" and "Not assigned/Function unknown".

The analysis of the top-100 genes with the highest level of expression at the exponential growth phase (Table S2) showed that the genes belonging to the functional category "Translation, ribosomal structure and biogenesis" were highly represented (46%), with the proportion of genes from the remainder functional categories never exceeding 15%. Also, 15 genes with unknown function were detected among the highly expressed genes (GBS_RS11490, GBS_RS06375, GBS_RS06390, GBS_RS06380, GBS_RS06405, GBS_RS06400, GBS_RS06385, GBS_RS06800, GBS_RS11205, GBS_RS03445, GBS_RS10615, GBS_RS06395, GBS_RS00250, GBS_RS05190, GBS_RS11525). Although these genes have not been grouped into any functional category by RPS-BLAST against the COG database, fine-tune evaluation of their putative function based on the new NEM316 genome annotation (released on June 2020), plus literature surveys, provided some clues that might justify the observed high expression level. This is the case of GBS_RS00250, which is believed to be required for S. agalactiae cell division due to its potential role in peptidoglycan cleavage, since it includes a CHAP domain that has been associated with peptidoglycan hydrolysis (23). Disruption of this gene was shown to cause an altered cell morphology and an increased susceptibility toward different antibiotics, namely β-lactam antibiotics (24, 25). GBS_RS05190, a PASTA domain-containing protein, may also be involved in bacterial cell division as PASTA repeats are known to be key regulators of the membrane during bacterial cell division (26). GBS_RS11205 is a putative holin-like toxin, and holins, which are encoded by phages, have been considered responsible for disruption of the cytoplasmic membrane to assist endolysins during cell lysis (27). Although most of these proteins are implicated in the bacterial stress response, like GBS_RS06375, GBS_RS06380, GBS_RS06405, GBS_RS06400, GBS_RS06385, GBS_RS03445 and GBS_RS06395 (10, 28-30), others (GBS_RS11490, GBS_RS06390, GBS_RS06800, GBS_RS10615, GBS_RS11525) do not have any assigned function, neither any predicted functional domain.

The functional category "Intracellular trafficking, secretion, and vesicular transport", which belongs to one of the functional categories with the lowest median expression levels, was also represented among the top-100 most expressed genes by GBS_RS09920 and GBS_RS00560 (preprotein translocase subunit YajC and preprotein translocase subunit SecY, respectively).

The functional category "Signal transduction mechanisms" was represented among the top-100 most expressed

genes by only one gene, GBS_RS08755, a response regulator transcription factor, CovR, known to play a role in virulence gene expression (31,32).

Finally, we were interested in assessing the expression levels of a selected set of genes potentially linked to virulence (n=47) (Figure 2). The set includes the seven genes that putatively encode for secreted DNases (GBS_RS03720, GBS_RS04825, GBS_RS01045, GBS_RS03490, GBS_RS02295, GBS_RS03960, GBS_RS05380), and the genetic pilus islands (PI) that consist of five genes encoding for pilus assembly (33), PI-1 (that plays an important role in evasion from host innate immunity) (GBS_RS03580, GBS_RS03575, GBS_RS03570, GBS_RS03585 and GBS_RS03565) and PI-2a (that is specifically involved in adhesion and biofilm formation) (GBS_RS07760, GBS_RS07745, GBS_RS07765, GBS_RS07755 and GBS_RS07750) (34, 35). The major nuclease, nuclease A (GBS_RS03720; old locus tag: gbs0661), identified in S. agalactiae NEM316 by Derré-Bobillot and co-authors (16), presented higher mean expression values (ranked at position 520º out of a total of 2169 genes evaluated) than the other DNase encoding genes (Figure 2). Among the PI genes, PI-2a presented higher mean expression values (ranked between positions 84º and 718º out of a total of 2169 genes evaluated) than PI-1 coding genes (ranked between positions 1058º and 1973º out of a total of 2169 genes evaluated) (Figure 2).

Interestingly, three genes associated with virulence were ranked within the top-100 most expressed genes, including the PI-2a pilus major subunit PilB (GBS_RS07760) (Figure 2). The other two genes code for adhesins associated with the glycolytic pathway for energy metabolism; they were GBS_RS09445 (Glyceraldehyde-3-Phosphate Dehydrogenase, GAPDH), which is also thought to be involved in macromolecular interactions and bacterial pathogenesis, and GBS_RS03485 (enolase), belonging to "Carbohydrate transport and metabolism" functional category (36, 37).

## NEM316 comparative transcriptomic analysis through exposure to DNA

The scientific interest in S. agalactiae extracellular nucleases increased with the discovery, by Brinkmann and colleagues in 2004 (38), that they can disrupt the DNA matrix, which constitutes the nuclear backbone of neutrophil extracellular traps (NETs). Indeed, as NETs (also composed of granule proteins and histones) (16, 39, 40) are released by neutrophils to degrade virulence factors and kill bacteria, this nuclease-mediated mechanism could play an important role in S. agalactiae virulence. Since then, although important knowledge has been acquired about S. agalactiae nucleases (4, 16, 17, 41, 42) very little was added to the knowledge of the complex molecular pathways and stimuli triggering the release of DNases in vitro and in vivo. Here, we conducted RNA-seq assays to understand whether direct exposure to DNA would constitute a stimulus that could affect gene expression during the exponential growth phase, by using reference strains NEM316, a recognized DNase producer. Although a preliminary assay at the transcriptomic level targeting gbs0661 revealed no significant expression differences among reference strains NEM316 (DNase producer) and 2603V/R (DNase non-producer) with and without DNA stimuli (data not shown), we hypothesized that exposure to human DNA could trigger differential gene expression in other S. agalactiae genes (potentially involved in molecular cascades mediating DNase release and virulence). However, this

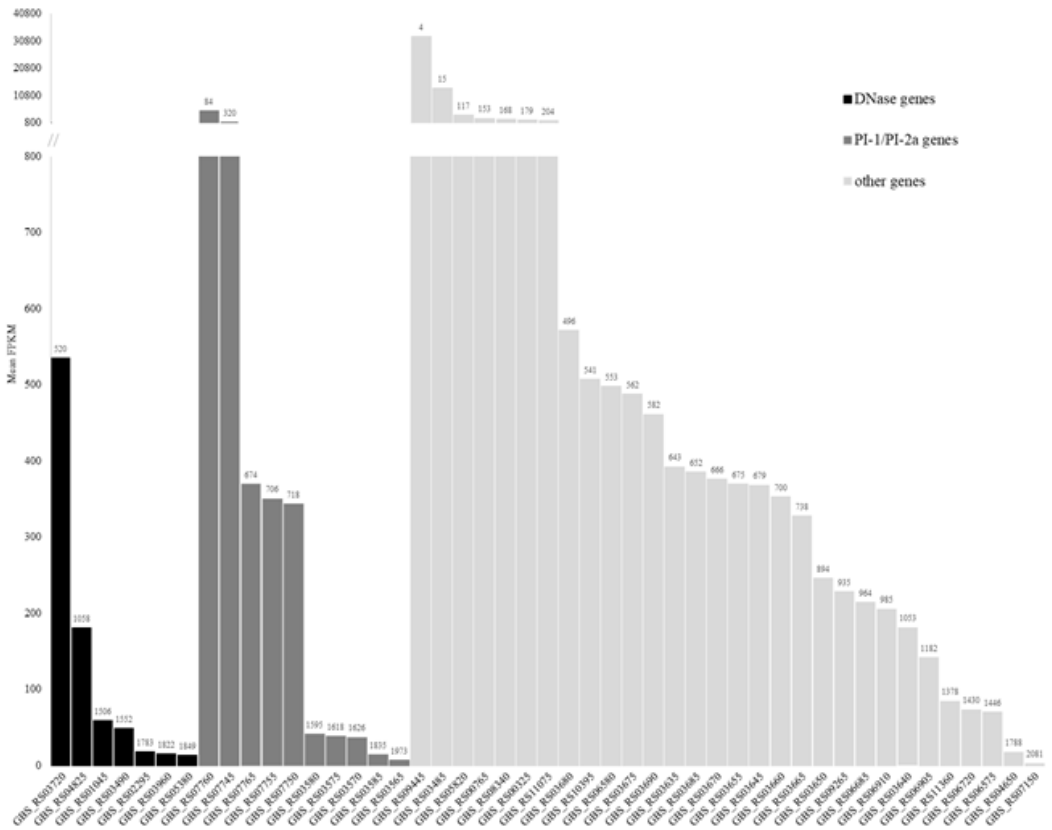


**Figure 2. Expression level (FPKM units) of genes potentially involved in virulence in reference strain NEM316 at exponential growth phase.** Genes are grouped according to their predictive function in three groups, DNase, PI-1/PI-2a and other coding genes, and displayed by increasing level of expression. The values above the bars correspond to the rank position of each gene in the total of 2169 genes evaluated.

hypothesis was not verified, as no differentially expressed genes were detected for NEM316 during 20 minutes of the exponential phase (at 37ºC in THB), either with or without the presence of human DNA (interactive online data navigation is available here: http://degust.erc.monash.edu/degust/compare.html?code=b11b5fff2bf525ea465dc450989f351e#). While this data could suggest that human DNA, as a stimulus, has no impact on S. agalactiae genetic transcription, other stimuli, as well as other strains, should be evaluated, as streptococcal DNases are under the control of the extensive regulatory systems (17). Thus, further work is required to fully understand the complex regulation of the expression of DNases, namely comparative RNA-seq assays involving both high and low DNase producing strains in comparison with DNase non-producers, as well as the use of S. agalactiae strains of different human clinical origin (e.g. carriage and invasion). In addition, human neutrophils and NETs might better mimic the in vivo infection environment than the exposure to DNA, and thus, their impact on gene expression can also be of interest in future evaluations. Finally, we cannot exclude the need of evaluating the intrinsic catalytic mechanism/activity of S. agalactiae DNases.

Despite the limitations, this pioneer assay allowed the public release of preliminary novel data (counts per million, logFC and differential expression statistics, etc.) to the scientific community through an interactive and user-friendly web tool (http://degust.erc.monash.edu/degust/compare.html?code=b11b5fff2bf525ea465dc450989f351e#). This should be of help for the analysis and interpretation of future RNA-seq studies in S. agalactiae, required to the disclosure of the complex molecular pathways (and putative stimuli) triggering the release of DNases.

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**Interest conflict**
The authors declare no conflict of interest/competing interests.

**Author's contribution**
All authors had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. MJB, JPG and VB conceptualized and supervised the study. IS performed research and the experimental procedures. SD performed the wet-lab RNA-seq procedures. IS and VB co-analyzed the RNA-seq data. The first draft of the manuscript was written by IS and all authors revised and commented on the manuscript. All authors read and approved the final manuscript.

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**Availability of data and material**
All data generated or analysed during this study are included in this published article and its supplementary information files. WGS and RNA-seq reads were deposited in the European Nucleotide Archive (ENA) under the Bioproject PRJEB41294.

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