Transcriptome Adaptation of Group B *Streptococcus* to Growth in Human Amniotic Fluid

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Abstract

**Background:** *Streptococcus agalactiae* (group B *Streptococcus*) is a bacterial pathogen that causes severe intrauterine infections leading to fetal morbidity and mortality. The pathogenesis of GBS infection in this environment is poorly understood, in part because we lack a detailed understanding of the adaptation of this pathogen to growth in amniotic fluid. To address this knowledge deficit, we characterized the transcriptome of GBS grown in human amniotic fluid (AF) and compared it with the transcriptome in rich laboratory medium.

**Methods:** GBS was grown in Todd Hewitt-yeast extract medium and human AF. Bacteria were collected at mid-logarithmic, late-logarithmic and stationary growth phase. We performed global expression microarray analysis using a custom-made Affymetrix GeneChip. The normalized hybridization values derived from three biological replicates at each growth point were obtained. AF/THY transcript ratios representing greater than a 2-fold change and P-value exceeding 0.05 were considered to be statistically significant.

**Principal Findings:** We have discovered that GBS significantly remodels its transcriptome in response to exposure to human amniotic fluid. GBS grew rapidly in human AF and did not exhibit a global stress response. The majority of changes in GBS transcripts in AF compared to THY medium were related to genes mediating metabolism of amino acids, carbohydrates, and nucleotides. The majority of the observed changes in transcripts affects genes involved in basic bacterial metabolism and is connected to AF composition and nutritional requirements of the bacterium. Importantly, the response to growth in human AF included significant changes in transcripts of multiple virulence genes such as adhesins, capsule, and hemolysin and IL-8 proteinase.

**Conclusions/Significance:** Our work provides extensive new information about how the transcriptome of GBS responds to growth in AF, and thus new leads for pathogenesis research.

Introduction

Intrauterine and postpartum infections remain an important cause of morbidity and mortality worldwide. One of the bacterial species commonly responsible for these infections is *Streptococcus agalactiae*, also known as group B *Streptococcus* (GBS). GBS colonizes the urogenital or gastrointestinal tract of about 10%–30% of humans, depending on gender, geographical origin, ethnicity, and screening method used (for a review see [1] and references therein). In recent decades GBS has become an important human pathogen [2], now responsible for a large percentage of female urogenital tract infections in non-pregnant women and ammonitosis and septic abortion in pregnant individuals [3,4]. GBS also is a major cause of fatal septicemia and meningitis in newborns and invasive infections in elderly and people with underlying diseases [3,4]. Recent studies [5,6,7] have shown that the transcriptome of GBS responds extensively to environmental changes, therefore we hypothesized that this organism will significantly remodel its global transcript profile in response to growth in human amniotic fluid. In this study, we employed an *ex vivo* strategy to characterize the global transcriptome response of GBS when grown in human amniotic fluid. The *ex vivo* strategy has been successfully used to study adaptation of pathogenic bacteria to multiple environments of the human body such as blood and saliva [7,8,9]. To gain significant new information about the interaction of GBS with amniotic fluid over time, we conducted expression microarray analysis at three time points throughout the pathogen growth cycle.

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Materials and Methods

Bacterial strains and routine growth

Serotype III GBS strain NEM316 was used in these studies because the genome has been sequenced; the organism has been used in many pathogenesis studies, and serotype III organisms cause a large number of serious human infections [10]. The strain was grown in Todd Hewitt medium with 0.5% yeast extract (THY) or on Trypticase Soy agar (TSA) II plates supplemented with 5% sheep blood (BD Diagnostics) at 37°C in a 5% CO2 atmosphere.

Growth of GBS in human amniotic fluid

Human amniotic fluid (AF) was collected from pregnant women seen at The Methodist Hospital, Houston, Texas, or Weill Medical College of Cornell University in New York City. Samples were collected in accordance with an exempt human subjects protocol approved by the Institutional Review Board of each institution. The study involved collection of existing diagnostic specimens routinely collected during clinical procedures as amniocenteses and would have been otherwise discarded. Specimens were stripped of all identifiers and processed in a manner that subjects cannot be directly or indirectly identified.

After collection, each specimen was centrifuged to remove host cells, filtered sterilized, and frozen at −20°C. After thawing, each AF sample was tested to determine if it supported growth of GBS. Aliquots (250 µl) of heat inactivated (95°C for 5 min) AF were inoculated with GBS prepared as follows. Bacteria from overnight cultures grown in THY were collected by centrifugation, washed twice with sterile PBS, and suspended in PBS. 10 µl of 100× bacteria diluted further in PBS were used to inoculate each 250 µl sample of AF (resulting in a final inoculum of ~10^4 CFU/ml) and were incubated at 37°C in 5% CO2 for 24 h. To avoid artifacts caused by carryover of THY medium, after 24 h of growth in AF (designated AF1), the GBS were diluted 1:500 into a fresh aliquot of AF (designated AF2). Growth of GBS in AF2 was quantified every hour for first 12 h and thereafter every 12 h by plating serial dilutions on TSA II plates (BD Diagnostics). For transcriptome studies, AF samples were pooled and three independent AF2 cultures were inoculated with GBS (biological replicates). Bacteria were collected by centrifugation at time points corresponding to the mid-logarithmic (ML), late-logarithmic/early stationary (LL) and stationary (S) phase of growth (Fig. 1).

Cultures grown in THY medium were prepared as described previously [5]. Briefly, three independent cultures of GBS were grown in the same lot of THY broth and GBS cells were harvested at three time points corresponding to mid-logarithmic (ML), late log/early stationary (ES), and stationary (S) growth phase (Fig. 1).

RNA isolation and processing

The bacterial aliquots used for RNA isolation were mixed with 2 volumes of RNA Protect reagent (Qiagen), and the cells were collected by centrifugation and stored at −80°C. RNA was isolated using a modified TRIZOL (Invitrogen) method [11]. Briefly, GBS pellets were suspended in 200 µl of Max Bacterial Enhancement Reagent (Invitrogen), incubated according to the manufacturer’s recommendations, mixed with 1 ml of TRIZOL, and disrupted using lysis matrix B (MP Biomedical). Cell debris was removed by centrifugation, and RNA was extracted with chloroform and precipitated with isopropanol. The precipitated RNA was suspended in 100 µl of RNase free water (Ambion, Austin TX) and further purified using RNeasy 96 well plates. All samples were processed simultaneously to minimize experimental variation. Reverse transcription, cDNA fragmentation, and labeling was performed as described previously [12].

Microarray analysis

Microarray analysis was performed using a custom-made Affymetrix chip that contained 1,994 probe sets, representing the annotated ORFs of GBS strain NEM316 [13]. Chip hybridization data were acquired using Affymetrix GeneChip Operating Software (GCOS 1.4) and normalized to allow multi-condition comparison. GCOS-acquired hybridization intensity values were normalized to the total intensity of all GBS genes present on the chip. Individual intensity for the gene transcripts generated by GCOS was divided by the sum of all intensities of the GBS hybridizing probes. Normalized hybridization values were used in all subsequent analysis. Data derived from three biological replicates obtained from three independent cultures were used to calculate mean values. PartekPro (Partek) and Array Assist (Stratagene) software were used to assess chip quality and chip-to-chip variability, and for data mining and visualization. Average normalized hybridization values were used to calculate AF/THY transcript ratios. Average values generated after hybridization of samples from AF_ML phase were divided by values generated previously.
from samples THY_ML to generate mid-logarithmic AF/THY ratios (ML). Average values from samples AF_LL were divided by values generated from samples THY_ES to generate late-logarithmic AF/THY ratios (LL). Average values from samples AF_S were divided by values generated from samples THY_S to generate stationary-phase AF/THY ratios (S). Only AF/THY ratios above a 2-fold change and with a P value less than 0.05 were included in the functional analysis. Normalized hybridization values are deposited in GEO database (http://www.ncbi.nlm.nih.gov/geo/) under GSE14456 and GSE12238 accession numbers.

Results and Discussion

Characterization of GBS growth in AF

Prior to characterizing the transcriptome of GBS grown in human AF, we studied the growth of strain NEM316 in THY
medium. GBS grew rapidly in this medium, with a generation time of \(\sim 35\) min in logarithmic phase (Fig. 1). The bacterial density reached \(\sim 10^9\) CFUs/ml in the stationary phase (Fig. 1). We next analyzed the growth of strain NEM316 in human AF. AF has been reported to have antimicrobial properties toward various species of bacteria due to \(\beta\)-lysin and lysozyme activity, which depends on divalent cations such as zinc and phosphate [14,15]. In multiple studies reported in the literature 18–73% of AF samples exhibited inhibitory properties towards various bacterial species. The antimicrobial properties depend on gestation stage and ethnicity [16,17,18,19,20]. Because individual specimens of AF can vary in their antimicrobial properties even towards GBS [21], we first tested the ability of the collected samples to support GBS growth. Consistent with the majority of the data reported in the literature, none of the AF specimens significantly inhibit the growth of strain NEM316 (data not shown). To avoid the effects of sample-to-sample variability, pooled AF was used in all subsequent experiments. Growth of GBS in AF is comparable with growth in THY with respect to cell density (\(\sim 10^9\) CFU/ml) and growth rate in exponential phase (Fig. 1).

Expression microarray analysis: quantitative differences during growth and in response to AF

To characterize the transcriptome of GBS grown in AF, we used an ex vivo expression microarray analysis strategy that we previously employed to study transcription interactions of streptococci with body fluids such as blood and saliva [7,8,9]. Strain NEM316 was grown in THY broth or pooled AF and harvested at various time points (Fig. 1). After transcriptome data acquisition, we assessed chip-to-chip data variability and quality using principal component analysis (PCA) (Fig. 2). The PCA analysis discriminated very well between the transcript data from the chips representing the various growth phases studied (Fig. 2). These results indicated that the transcriptome profile data from triplicate experiments were highly reproducible and of sufficient quality to permit robust statistical analysis and interpretation. The data clearly show that the transcriptome of GBS strain NEM316 is considerably remodelled in a growth-phase and growth medium-specific fashion. During growth of GBS in either THY [5] or AF (this work), we observed over 70% of all transcripts (table S1) exhibiting differential expression during at least one experimental growth phase what is a sign of great transcriptome plasticity in response to environment changing over time. Interestingly the biggest differences between expression in AF and THY are observed during transition from logarithmic to stationary phase (LL) (Fig. 3) and as many as 54% of all GBS transcripts present on the array are differentially expressed.

We next used a scatter plot analysis (Fig. 4) to compare the dynamics of transcript expression in ML, LL, and S phase between strain NEM316 grown in AF and THY. In the ML phase, transcript changes were rather modest, comparing with changes in LL and S phases, with AF/THY ratios rarely exceeding 10-fold. In the ML and LL phase transcripts are shifted toward THY, with a smaller number of transcripts expressed better in AF. In the S phase of growth, the general level of transcription is lower (note shift of spots towards left bottom corner, Fig. 4) than in ML and LL phase. However, the number of genes up regulated in AF is higher than in other phases of growth.

For ease of analysis and description, we assigned the GBS genes to functional categories based on their annotation and presumed involvement in metabolic processes or cell maintenance functions (Tables 1, 2, 3, 4, 5, 6 and Table S1). In general, the transcript levels of most genes in each functional category were better expressed during growth in THY. However, the transcripts of genes belonging to several functional categories, including amino acid, carbohydrate, and nucleotide metabolism were higher in AF at one or more growth points (Fig. 5A).

**Figure 3. Quantitative differences in gene expression between THY and AF.** Genes regarded as differentially expressed have an AF/THY transcript level ratio of 2 and above (better expressed in AF) or 0.5 and below (better expressed in THY). Arrow up, number of genes better expressed in AF; arrow down, number of genes better expressed in THY. ML, mid-logarithmic growth phase; LL, late-logarithmic growth phase; ES, early stationary growth phase; and S, stationary growth phase. doi:10.1371/journal.pone.0006114.g003

**Stress response to AF**

We hypothesized that growth in amniotic fluid will trigger expression of genes involved in adaptation and the stress response. Although we observed changes in the transcript levels of multiple genes involved in adaptation, protein secretion and trafficking, and DNA repair, surprisingly, the transcripts were down regulated in response to growth in AF (Table 1). The most striking examples are genes encoding putative cold shock protein (gb\(\mid\)2033, 55× down regulated in AF) or universal stress protein family (gb\(\mid\)1778, 27× down regulated in AF). However, we also observed moderate up-regulation of \(\text{gbEL}\) and \(\text{clpL}\) transcripts. Thus, it appears that GBS does not exhibit a classic stress response when grown in AF, but rather readily adapts to this environment. We also did not observe massive down regulation of protein synthesis as an effect of stress, and interestingly, production of some ribosomal protein transcripts in LL and S phase was higher in AF than in THY (Table S1).

**Regulatory events during growth in AF**

The lack of alternative sigma factors in GBS [10] means that a successful regulatory response to environmental changes relies mainly on differential transcription of genes encoding two component systems (TCS) and stand-alone regulators. The GBS genome has genes encoding multiple TCS systems that might be involved in adaptation to various environments. As expected, we observed differential expression of multiple TCS and putative regulators of unknown function (Table 2). One of the regulators
Figure 4. Dynamics of gene expression between THY and AF at various stages of growth. The location of each dot represents a single transcript. Dotted lines denote two-fold difference between transcript level in AF and THY, thick lines denote ten-fold difference in expression between AF and THY. Each panel represents differences in expression in mid-logarithmic (ML), late-logarithmic (LL) and stationary (S) growth phases. x-axis, expression level in THY; y-axis, expression level in AF.
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with the greatest degree of transcriptional change encodes carbon starvation protein A (Table 2). This change, together with observed alteration in transcripts of the genes encoding carbon catabolite proteins A and B might contribute to the large number of differential transcripts observed in genes encoding metabolic proteins (see below). In addition, we observed lowered expression of codY, a regulator involved predominantly in amino acid metabolism and activated by branched chain amino acids [22]. In *Streptococcus pneumoniae* inactivation of codY gene is linked to decreased expression of pcpA adhesin and lower adhesion to human cells in vitro, suggesting possible mechanism linking metabolic state of the bacterium and pathogenic properties [23]. Recent analysis of *Streptococcus pyogenes* adaptation to blood found that a codY mutant strain strongly up-regulated expression of genes encoding branched chain amino acids transporters [24], an observation consistent with differential expression of branched chain amino acid transporter genes we found in our experiment.

| Locus     | Name    | ML  | LL  | S   | Putative function                        |
|-----------|---------|-----|-----|-----|------------------------------------------|
| gbs0009   | -       | 7.2 | -   | -   | Heat shock protein 15                     |
| gbs0015   | ftsH    | -   | 9.0 | -   | Cell division protein ftsH (EC 3.4.24.-)  |
| gbs0095   | grpE    | -   | 3.7 | -   | GrpE protein                             |
| gbs0097   | dnaJ    | -   | 6.3 | -   | chaperone protein                        |
| gbs0104   | tig     | -   | 5.3 | -   | Trigger factor, ppase (EC 5.2.1.8)        |
| gbs0109   | radA    | -   | 8.5 | -   | DNA repair protein RadA                   |
| gbs0222   | -       | 3.2 | -   | 3.1 | DNA-damage-inducible protein J            |
| gbs0284   | -       | -   | 2.3 | -   | Thioredoxin reductase (EC 1.8.1.9)        |
| gbs0289   | recU    | -   | 14.7| -   | Recombination protein recU                |
| gbs0447   | recX    | -   | 6.3 | -   | Regulatory protein recX                   |
| gbs0502   | -       | -   | 5.0 | -   | ATP-dependent endopeptidase Lon (EC 3.4.21.53) |
| gbs0547   | recN    | -   | 2.4 | -   | DNA repair protein recN                    |
| gbs0786   | recR    | -   | 2.1 | 4.3 | Recombination protein recR                 |
| gbs0838   | nrdH    | -   | 5.6 | -   | Glutaredoxin                              |
| gbs1202   | -       | -   | 2.4 | -   | General stress protein, Gls24 family      |
| gbs1257   | clpL    | -   | 2.6 | 5.2 | ATP-dependent endopeptidase clp ATP-binding subunit clpL |
| gbs1383   | clpX    | -   | 3.9 | -   | ATP-dependent endopeptidase clp ATP-binding subunit clpX |
| gbs1423   | trxB    | -   | 2.6 | -   | Thioredoxin reductase (EC 1.8.1.9)        |
| gbs1586   | -       | 3.6 | -   | 2.4 | Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8) |
| gbs1674   | -       | 2.4 | 2.9 | -   | Endopeptidase htpX (EC 3.4.24.-)          |
| gbs1721   | -       | -   | 2.3 | 2.2 | Universal stress protein family           |
| gbs1738   | -       | -   | 2.1 | -   | General stress protein, Gls24 family      |
| gbs1764   | mutS2   | -   | 2.8 | -   | DNA mismatch repair protein mutS           |
| gbs1778   | -       | -   | 14.2| 27.0| Universal stress protein family           |
| gbs1865   | hsIO    | -   | 8.0 | -   | 33 kDa chaperonin                         |
| gbs2029   | groEL   | 2.2 | 2.3 | 2.0 | 60 kDa chaperonin GROEL                   |
| gbs2048   | cinA    | -   | -   | 2.3 | competence/damage-inducible protein CinA  |
| gbs2052   | mutL    | -   | 2.4 | -   | DNA mismatch repair protein mutL          |
| gbs2053   | csp     | -   | 55.0| 6.0 | Cold shock protein                        |
| gbs2054   | mutS    | -   | 3.1 | -   | DNA mismatch repair protein mutS           |
| gbs2113   | -       | -   | 4.8 | -   | Non-proteolytic protein, peptidase family M16 |
| gbs2115   | recF    | -   | 4.9 | -   | DNA replication and repair protein recF    |

Values represent fold change in expression in amniotic fluid compared to expression in THY; ML, mid-logarithmic growth phase; LL, late-logarithmic growth phase; S, stationary growth phase; cut-off two fold change with P value less than 0.05. Positive values represent genes up-regulated in AF, negative values represent down-regulated (better expressed in THY) genes. Full list of changes is published as Table S1. doi:10.1371/journal.pone.0006114.t001
Expression of virulence factors

Compared to other pathogenic streptococci such as GAS, virulence factors of GBS are much less studied and therefore not well understood. However, we observed differential expression of multiple putative cell wall anchored proteins (Table 3) and proven virulence factors. For example, the cgl operon, encoding a hemolysin, required for survival in blood and under oxidative stress [27,28] was up-regulated during growth in human AF. One of the more striking observations was very high up-regulation of the homolog of GAS SpyCEP (gbs2008). This extracellular protease cleaves and inactivates human interleukin 8 and contributes to virulence in GBS [29] and GAS [30,31]. In GAS, SpyCEP is greatly up-regulated in strains causing invasive infections compared to those recovered from patients with superficial infections such as pharyngitis [31]. We also detected an increased level of transcripts encoded by genes located in putative pathogenicity island IX (gbs1061-gbs1076), function of this element is unknown. Interestingly, transcripts of genes

| Locus     | Name     | ML   | LL   | S    | Putative function                              |
|-----------|----------|------|------|------|------------------------------------------------|
| gbs0094   | hrcA     | −2.2 | −2.2 | −2.2 | Heat-inducible transcription repressor hrcA    |
| gbs0105   | rpoE     | −17.7| −17.7| −17.7| DNA-directed RNA polymerase delta chain (EC 2.7.7.6) |
| gbs0121   | mecA     | −2.3 | −2.3 | −2.3 | Two-component response regulator               |
| gbs0135   | rpoB     | −17.4| −17.4| −17.4| DNA-directed RNA polymerase beta chain (EC 2.7.7.6) |
| gbs0181   | -        | −3.9 | −3.9 | −3.9 | Autolysin response regulator                   |
| gbs0248   | -        | −2.3 | −2.3 | −2.3 | ECF-type sigma factor negative effector        |
| gbs0249   | -        | −3.4 | −3.4 | −3.4 | RNA polymerase ECF-type sigma factor           |
| gbs0299   | -        | −4.4 | −4.4 | −4.4 | Two-component response regulator               |
| gbs0302   | -        | −9.2 | −9.2 | −9.2 | DNA-directed RNA polymerase omega chain (EC 2.7.7.6) |
| gbs0414   | nusA     | −2.6 | −2.6 | −2.6 | transcription elongation factor NusA           |
| gbs0427   | perR     | −2.9 | −2.9 | −2.9 | Oxidative stress response regulator BosR        |
| gbs0546   | argR1    | −4.0 | −4.0 | −4.0 | Arginine repressor, argR                       |
| gbs0580   | rpoB     | −2.6 | −2.6 | −2.6 | DNA-directed RNA polymerase rpoB               |
| gbs0741   | vicR     | −2.2 | −2.2 | −2.2 | Two-component response regulator VicR          |
| gbs0756   | -        | −3.5 | −3.5 | −3.5 | Stress-responsive transcriptional regulator PspC |
| gbs0804   | -        | −4.3 | −4.3 | −4.3 | Catabolite control protein A                   |
| gbs1050   | -        | −4.4 | −4.4 | −4.4 | Carbon starvation protein A                    |
| gbs1398   | -        | −6.8 | −6.8 | −6.8 | Two-component response regulator               |
| gbs1496   | rpoD     | −2.8 | −2.8 | −2.8 | RNA polymerase sigma factor rpoD               |
| gbs1530   | rpfA     | −5.3 | −5.3 | −5.3 | transcriptional regulator                     |
| gbs1672   | covR     | −2.3 | −2.3 | −2.3 | Response regulator CsrR                       |
| gbs1719   | codY     | −11.0| −11.0| −11.0| Transcriptional regulatory protein codY         |
| gbs1736   | scrR     | −4.3 | −4.3 | −4.3 | Sucrose operon repressor                      |
| gbs1835   | -        | −2.6 | −2.6 | −2.6 | Transcriptional regulatory protein             |
| gbs1870   | ctsR     | −4.7 | −4.7 | −4.7 | Transcriptional regulator ctsR                |
| gbs1882   | -        | −2.4 | −2.4 | −2.4 | Catabolite gene activator                     |
| gbs1909   | dpiA     | −18.6| −18.6| −18.6| Transcriptional regulatory protein             |
| gbs1934   | -        | −2.7 | −2.7 | −2.7 | Two-component response regulator yesN         |
| gbs1944   | fasA     | 2.0  | 2.0  | 2.0  | Response regulator FasA                       |
| gbs1948   | -        | −2.6 | −2.6 | −2.6 | Alkaline phosphatase synthesis two-component response regulator phoP |
| gbs2055   | argR2    | −4.0 | −4.0 | −4.0 | Arginine repressor, argR                       |
| gbs2081   | -        | −2.3 | −2.3 | −2.3 | Transcriptional regulatory protein             |
| gbs2087   | -        | −10.8| −10.8| −10.8| Two-component response regulator               |
| gbs2119   | ahcC2    | −3.3 | −3.3 | −3.3 | Arginine repressor, argR                       |

Values represent fold change in expression in amniotic fluid in comparison with expression in THY. Positive values represent genes up-regulated in AF, negative values represent down-regulated (better expressed in THY) genes. ML, mid-logarithmic growth phase; LL, late-logarithmic growth phase; S, stationary growth phase; cut-off: fold change above 2 and P value less than 0.05. Full list of changes is published as Table S1.

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| Locus    | Name | ML | LL | S  | Region | Putative function                      |
|----------|------|----|----|----|--------|----------------------------------------|
| gbs0031  |      |    |    |    |        | Surface antigen                        |
| gbs0393  |      | −3.5| −4.2| −3.2|        | Hypothetical protein                    |
| gbs0451  |      | 3.2 | −2.6|     |        | C5a peptidase precursor                |
| gbs0470  | alp2 | −2.3| −2.8|    |        | Cell surface protein                   |
| gbs0631  |      |    |    | 2.9 |        | Sortase                                |
| gbs0644  | cylX |    | 4.6 | H  |        | Hypothetical protein                    |
| gbs0645  | cylD | 2.6 | 5.5 | H  |        | Malonyl-CoA-transacylase                |
| gbs0646  | cylG | 3.7 | 4.8 | H  |        | 3-oxoacyl-reductase                    |
| gbs0647  | acpC | 3.9 | 4.3 | H  |        | (3R)-hydroxymyristoyl-dehydratase      |
| gbs0648  | cylA | 2.7 | 4.0 | H  |        | ATP-binding protein                    |
| gbs0650  | cylB | 3.6 | 2.5 | H  |        | Permease                               |
| gbs0651  | cylE | 3.4 | 2.3 | H  |        | Hypothetical protein                    |
| gbs0652  | cylF | 3.8 | 2.4 | H  |        | Aminomethyltransferase                 |
| gbs0653  | cylI | 4.0 | 2.3 | H  |        | 3-oxoacyl synthase                     |
| gbs0654  | cylJ | 3.9 | 2.2 | H  |        | UDP glycosyltransferase                |
| gbs0655  | cylK | 2.7 | 2.1 | −2.5| H      | Hypothetical protein                    |
| gbs0656  |      |    |    | 5.1 |        | Hypothetical protein                    |
| gbs1061  |      | 4.1 | I  |    |        | Hypothetical protein                    |
| gbs1062  |      | 3.8 | I  |    |        | Hypothetical protein                    |
| gbs1064  |      | 2.3 | I  |    |        | Hypothetical protein                    |
| gbs1065  |      | 2.6 | I  |    |        | Hypothetical protein                    |
| gbs1066  |      | 3.5 | I  |    |        | Hypothetical protein                    |
| gbs1067  |      | 3.2 | I  |    |        | Hypothetical protein                    |
| gbs1068  |      | 3.2 | I  |    |        | DNA segregation ATPase                 |
| gbs1069  |      | 5.7 | I  |    |        | Hypothetical protein                    |
| gbs1070  |      | 5.1 | 3.9| 5.7 | I      | Hypothetical protein                    |
| gbs1071  |      | 4.8 | 3.8| I   |        | Hypothetical protein                    |
| gbs1072  |      | 4.7 | 4.5| I   |        | Hypothetical protein                    |
| gbs1073  |      | 4.1 | 3.3| I   |        | Phage infection protein                |
| gbs1074  |      | 4.6 | 4.8| I   |        | Hypothetical protein                    |
| gbs1075  |      | 4.9 | 7.5| 3.9 | I      | Hypothetical protein                    |
| gbs1076  |      | 3.8 | 6.5| 7.0 | I      | Hypothetical protein                    |
| gbs1087  |      | 3.5 | 3.0|     |        | Hypothetical protein                    |
| gbs1104  |      | −2.1|    |    |        | Antigen                                |
| gbs1143  | epf | −3.0| −5.4|    |        | Cell surface protein                    |
| gbs1144  |      | −2.3| −2.2| C  |        | Cell surface protein                    |
| gbs1234  | neuD |    |    | 2.7 | C      | Sialic acid biosynthesis protein NeuD   |
| gbs1237.1| cpsL | −2.2|     | C  |        | beta-D-Galp alpha-2,3-sialyltransferase |
| gbs1238  | CpslA| −2.5|     | C  |        | beta-D-GlcNAc beta-1,4-galactosyltransferase |
| gbs1239  | hasA | −5.0|     | C  |        | beta-D-Galp beta-1,3-N-acetylgalcosaminyltransferase |
| gbs1240  | cpsI | −2.5| −4.9| C  |        | Secreted polysaccharide polymerase     |
| gbs1241  | cpsG | −3.2|     | C  |        | beta-D-Glc beta-1,4-galactosyltransferase |
| gbs1242  | cpsF | −3.2|     | C  |        | Beta-1,4-galactosyltransferase accessory protein |
| gbs1248  | cpsY | −2.2| −2.5| C  |        | Transcriptional regulators, LysR family |
| gbs1307  | Imb | −8.5|     |     |        | Laminin-binding surface protein        |
| gbs1356  |      |    |    | 8.1 |        | Cell surface protein                    |
| gbs1403  |      | 8.1 |    |    |        | S -nucleotidase                        |
| gbs1420  |      | −2.8|    |    |        | Choline-binding protein                 |
| gbs1474  |      | −3.6| −2.6|    |        | Hypothetical protein                    |
encoding multiple proteins implicated in adhesion to host molecules such as fibronectin, collagen, and laminin were significantly down-regulated during growth in AF. The C5a peptidase gene transcript also was significantly lower in GBS grown in AF, consistent with its involvement in fibronectin binding [32]. Down-regulation of adhesins seems to be consistent with increased virulence. For example, molecular epidemiological data suggest a negative correlation between binding of fibronectin and severity of GAS infection [33]. Over-expression of fibronectin binding protein decreases the virulence of GAS lacking fibronectin binding protein gene resulted in decreased virulence and lack of fibronectin binding protein the surface promotes bacterial dissemination [34].

Metabolism: Nutrient acquisition and energy production

GBS requires multiple exogenous compounds for growth, especially amino acids (AA). AF is composed mostly of water, urea, small amounts of amino acids, keratin from shed host epithelial cells, and proteins [35,36,37]. Thus, AF is relatively poor in nutrients, which means that bacteria with complex nutritional requirements will not grow or grow poorly. Unexpectedly, strain NEM316 grew very rapidly and to high cell density in AF (Fig. 1). We discovered that genes encoding systems that facilitate transport of amino acids and peptides were prominently up-regulated when GBS was grown in AF (Figure 5AB). In particular, multiple transport systems for branched-chain amino acids (isoleucine, leucine, valine) were very highly up-regulated, on the order of up to 500-fold more highly expressed during growth in THY (Table 4). Recently Samen and co-workers [38] showed that growth of GBS in AF depends on intact isoleucine and oligopeptide transport systems. Because GBS is auxotrophic towards multiple amino acids, presumably the up-regulation of amino acid transport systems is a direct result of an effort to scavenge these molecules. Oligopeptide transport systems in group A and B streptococci has been also shown to be involved in pathogenic properties as adhesion [38,39]. Recently, increasing number of reports links ability to utilize nutrients and metabolic state of the bacterium with its pathogenic properties [13,40]. Therefore, similar to carbohydrates, amino acid and oligopeptide transport and utilization processes might play a role in pathogenicity of GBS.

We also observed significant differential expression of genes in the arginine deiminase pathway. Arginine fermentation can be used for energy production by GAS, and likely GBS [41]. The genome of GBS strain NEM316 has two putative sets of genes involved in this metabolic pathway. However, it has not been confirmed experimentally if both of them are indeed involved in arginine deiminase pathway. Locus 1 (with high homology to GAS) is up regulated in response to AF, locus 2 with lower homology to GAS genes is down regulated in response to AF. Arginine deiminase seems to have a profound effect on streptococcal biology and virulence. We recently described regulation of arginine deiminase by growth phase [5] in GBS, and a similar phenomenon was also described recently for Streptococcus gordonii [42]. Arginine deiminase also influences expression of fimbrin in Porphyromonas gingivalis [43]. In GAS, arginine utilization is under control of major regulators rgg [44] and cfpA [13] and arginine deiminase is a potent inhibitor of human T-cell proliferation [45]. Moreover, despite the fact that it lacks export signal sequence, arginine deiminase is found on the GAS cell surface [46] and is a protective antigen in mice [A. Henningham, M.R Batzloff, J.C. Cole, C.M. Gillen, J. Hartas, K. S. Sripromak, M. J. Walker, poster nr. P34, 2008 Lancefield International Symposium on Streptococci & Streptococcal Diseases, Porto Heli, Greece].

Additionally, arginine metabolism is linked to nucleotide metabolism by carbamoyl phosphate. The carAB genes encoding subunits of carbamoyl phosphate synthase are also differentially regulated in GAS grown in AF. We observed significant up regulation of gene transcripts for almost all enzymes involved in de novo purine and pyrimidine synthesis and down regulation of salvage pathways (Table 5). This was especially prominent in the early stages of growth, suggesting that AF lacks sufficient free nucleotides for rapid growth.

Carbohydrate metabolism

Among genes involved in carbohydrate utilization in GBS, large portion is constituted by multiple transport systems (mostly PTS) that allow uptake of various carbohydrate sources. We detected differential expression of many genes involved in carbohydrate transport and metabolism (Table 6) (Fig. 5B). Interestingly, GBS
rather down-regulated genes responsible for the transport of simple carbohydrates what suggests their low concentration in AF.

### Summary

We have discovered that GBS significantly remodels its transcriptome in response to exposure to human amniotic fluid. A large number of the affected genes are of unknown function, which means that much remains to be learned about the full influence of amniotic fluid on GBS. The majority of the observed changes in transcripts affects genes involved in basic bacterial metabolism and is connected to AF composition and nutritional requirements of the bacterium. The observation that many genes encoding adhesions are down-regulated, and genes encoding known virulence factors such as a hemolysin and a potent IL-8

| GBS | Locus | ML  | LL  | S   | Descriptions |
|-----|-------|-----|-----|-----|--------------|
|     |       | 3.0 | 4.9 | 5.9 | Peptidases   |
| gbs0287 pepC | 3.0 | 4.9 | 5.9 | Peptidases | Aminopeptidase C (EC 3.4.22.40) |
| gbs121 pepN | 2.5 | 4.3 | Trieptidase T (EC 3.4.11.4) |
| gbs1459 pepT | 2.0 | 3.7 | Xaa-Pro dipeptidase (EC 3.4.13.9) |
| gbs1781 pepXP | 2.7 | Xaa-Pro dipeptidyl-peptidase (EC 3.4.14.11) |
| gbs0144 oppA | 2.4 | 2.6 | 3.5 | Oligopeptide transport system |
| gbs0146 oppC | 6.1 | Oligopeptide transport system |
| gbs0147 oppD | 2.2 | 9.0 | 4.1 | Oligopeptide transport system |
| gbs0148 oppF | 2.1 | 9.7 | 2.6 | Oligopeptide transport system |
| gbs0966 oppA | 23.2 | Oligopeptide-binding protein oppA |
| gbs1513 braB | 5.77 | Branched-chain amino acid transport |
| gbs1077 carB | 6.0 | 7.3 | Carbamoyl-phosphate synthase large chain |
| gbs1078 carA | 6.3 | 7.5 | Carbamoyl-phosphate synthase small chain |
| gbs2122 arcA | 2.7 | 2.1 | Arginine deiminase (EC 3.5.3.6) |
| gbs2123 arcB | 5.3 | Ornithine carbamoyltransferase (EC 2.1.3.3) |
| gbs2125 arcC | - | 5.5 | Arginine ornithine antiporter |
| gbs2126 arcD | -2.1 | 3.6 | Carbamate kinase (EC 2.7.2.2) |
| gbs2083 arcE | -7.5 | -140.4 | -31.7 | Arginine ornithine antiporter |
| gbs2084 arcF | -3.6 | -75.0 | -43.6 | Carbamate kinase |
| gbs2085 arcG | -2.3 | -28.8 | -19.7 | Ornithine carbamoyltransferase |

Values represent fold change in expression in amniotic fluid compared to expression in THY; ML, mid-logarithmic growth phase; LL, late-logarithmic growth phase; S, stationary growth phase; cut-off two fold change with P value less than 0.05. Positive values represent genes up-regulated in AF, negative values represent down-regulated (better expressed in THY) genes. Full list of changes is published as Table S1.
proteinase are up-regulated likely have consequences for the outcome of host-pathogen interactions.

Supporting Information

Table S1 Changes in transcription of GBS genes upon contact with amniotic fluid. All changes detected in transcription of GBS in response to amniotic fluid Values represent fold change in expression in amniotic fluid compared to expression in THY; ML, mid-logarithmic growth phase; LL, late-logarithmic growth phase; S, stationary growth phase; cut-off two fold change with P value less than 0.05. Positive values represent genes up-regulated in AF, negative values represent down-regulated (better expressed in THY) genes. Full list of changes is published as Table S1.

Values represent fold change in expression in amniotic fluid compared to expression in THY; ML, mid-logarithmic growth phase; LL, late-logarithmic growth phase; S, stationary growth phase; cut-off two fold change with P value less than 0.05. Positive values represent genes up-regulated in AF, negative values represent down-regulated (better expressed in THY) genes. Full list of changes is published as Table S1.

Adaptation of GBS to AF

Table 5. Selected genes involved in nucleotide metabolism regulated in response to amniotic fluid.

| GBS Locus | ML 2-fold change | LL 2-fold change | S 2-fold change | Descriptions |
|-----------|------------------|------------------|----------------|--------------|
| gbs0014   | -2.8             | 8.2              |                | Hypoxanthine-guanine phosphoribosyltransferase |
| gbs0017   | -6.1             | -2.8             |                | Ribose-phosphate pyrophosphokinase |
| gbs0023   | 32.8             | 28.0             |                | Phosphoribosylaminomimidazole-succinocarboxamidase synthase |
| gbs0024   | 38.9             | 56.1             |                | Phosphoribosylformylglycinamidine synthase |
| gbs0025   | 25.1             | 30.7             |                | Amidophosphoribosyltransferase |
| gbs0026   | 25.6             | 31.8             | 5.4            | Phosphoribosylformylglycinamidine cyclo-ligase |
| gbs0027   | 32.0             | 41.8             |                | Phosphoribosylglycinamidine formyltransferase |
| gbs0028   | 21.0             | 26.8             | 9.1            | Zwittermicin A resistance protein zmaR |
| gbs0029   | 27.6             | 29.3             | 12.4           | Phosphoribosylaminomimidazolecarboxamide formyltransferase |
| gbs0042   | 36.4             | 30.0             |                | Phosphoribosylamine-glycine ligase |
| gbs0043   | 49.0             | 47.5             |                | Phosphoribosylaminomimidazole carboxylase carboxyltransferase subunit |
| gbs0044   | 34.9             | 27.2             |                | Phosphoribosylaminomimidazole carboxylase NCAIR mutase subunit |
| gbs0047   | 3.0              | 3.6              |                | Adenylosuccinate lyase |
| gbs0106   | -                | -12.0            |                | CTP synthase [EC 6.3.4.2] |
| gbs0553   | 6.5              | 3.5              | 7.1            | Dihydroorotate dehydrogenase [EC 1.3.1.1] |
| gbs0558   | -6.5             | -                | 7.1            | dGTP triphosphohydrolase |
| gbs0574   | 7.3              | -                | 12.4           | Inosine-uridine preferring nucleoside hydrolase [EC 3.2.2.1] |
| gbs0583   | 4.4              | -                | 12.4           | Adenosine deaminase |
| gbs0836   | 32.2             | 3.2              |                | Ribonucleoside-diphosphate reductase beta chain |
| gbs0837   | 3.3              | -                | 12.4           | Ribonucleoside-diphosphate reductase alpha chain |
| gbs0844   | -29.6            | -                | 12.4           | Uridine kinase |
| gbs1079   | 7.1              | 4.5              |                | Aspartate carbamoyltransferase |
| gbs1080   | 3.7              | 3.6              |                | Dihydroorotase |
| gbs1081   | 4.7              | 2.7              |                | Orotate phosphoribosyltransferase |
| gbs1082   | 5.6              | 2.3              | 3.9            | Orotidine 5-phosphate decarboxylase |
| gbs1089   | 3.1              | 4.1              |                | Formate-tetrahydrofolic ligase |
| gbs1110   | -                | 5.0              |                | Thymidine kinase |
| gbs1116   | 2.9              | 2.6              |                | Xanthine permease |
| gbs1117   | 3.1              |                | 2.6            | Xanthine phosphoribosyltransferase |
| gbs1154   | 2.4              | 8.8              |                | GMP reductase |
| gbs1162   | -                | -6.0             | 4.1            | GTP pyrophosphokinase homolog |
| gbs1231   | -                | 2.7              |                | Uracil-DNA glycosylase |
| gbs1867   | -                | -13.0            |                | Deoxyadenosine kinase |
| gbs1929   | 11.7             | 2.7              | -3.1           | 2, 3-cyclic-nucleotide 2-phosphodiesterase |

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Author Contributions

Conceived and designed the experiments: IS NMG JMM. Performed the experiments: IS NG. Analyzed the data: IS NMG JMM. Contributed reagents/materials/analysis tools: AMB SW. Wrote the paper: IS JMM.
| Locus     | Name        | ML  | LL  | S    | Putative function                                      |
|-----------|-------------|-----|-----|------|--------------------------------------------------------|
| gbs0113   |             | -2.5|     | 9.5  | D-ribose-binding protein                                |
| gbs0114   |             | -2.3| 2.1 | 9.7  | Ribose transport system permease protein rbsC          |
| gbs0115   |             | 2.7 | 5.7 |      | Ribose transport ATP-binding protein rbsA              |
| gbs0116   |             | 2.4 | 6.2 |      | D-ribose mutarotase                                    |
| gbs0117   |             | 2.5 | 3.0 |      | Ribokinase                                             |
| gbs0316   | manN        | -   | 2.2 | 4.2  | PTS system, cellobiose-specific IIA component          |
| gbs0317   | manM        | -   | 3.0 |      | PTS system, cellobiose-specific IIC component          |
| gbs0346   |             | 2.2 | 2.7 | 4.2  | PTS system, mannose-specific IIB component             |
| gbs0347   |             | 2.1 | 2.0 | 2.2  | PTS system, mannose-specific IIAB component            |
| gbs0348   |             | 2.1 | 2.0 | 2.2  | PTS system, mannose-specific IIA component             |
| gbs0872   | glgC        | -   | 2.5 |      | Glucose-1-phosphate adenylyltransferase catalytic subunit |
| gbs0873   |             | -   | 3.0 |      | Glucose-1-phosphate adenylyltransferase regulatory subunit |
| gbs1329   | lacG        | -   | -17.1| 2.3  | 6-phospho-beta-galactosidase                           |
| gbs1330   | lacE        | -   | -8.1 |      | PTS system, lactose-specific IIIB component            |
| gbs1331   | lacF        | -   | -11.7 |     | PTS system, lactose-specific IIAC component            |
| gbs1333   | lacD.2      | -   | -12.7 |     | Tagatose-bisphosphate aldolase                        |
| gbs1334   | lacC.2      | -   | -2.8 | -18.7| Tagatose-6-phosphate kinase                            |
| gbs1335   | lacB.1      | -   | -11.2| 3.9  | Galactose-6-phosphate isomerase lacB subunit           |
| gbs1336   | lacA.2      | -   | -2.1 | -11.2| Galactose-6-phosphate isomerase lacA subunit           |
| gbs1507   | glgP        | -   | -2.4 | 5.1  | Maltodextrin phosphorylase                            |
| gbs1508   | malM        | -   | -2.9 | 5.9  | 4-alpha-glucanotransferase                             |
| gbs1510   | malE        | -   | 3.8  |      | Maltose maltodextrin-binding protein                    |
| gbs1511   | malF        | -   | 4.6  |      | Maltodextrin transport system permease protein malF     |
| gbs1512   | malG        | -   | 3.5  |      | Maltose transport system permease protein malG          |
| gbs1692   |             | -2.7|     |      | Dihydroxyacetone kinase                                |
| gbs1694   |             | -2.5| 5.1  | 5.9  | Dihydroxyacetone kinase                                |
| gbs1695   |             | 3.9 | 8.4  |      | Dihydroxyacetone kinase                                |
| gbs1696   |             | 2.6 | 14.5 |      | Dihydroxyacetone kinase                                |
| gbs1697   |             | -2.4| 2.5  | 11.8 | Glycerol uptake facilitator protein                     |
| gbs1714   |             | -   | 3.9  |      | Pyruvate-phosphate dikinase                            |
| gbs1732   | pmi         | -   | -3.2 |      | Mannose-6-phosphate isomerase                          |
| gbs1733   | scrK        | -6.9|     |      | Fructokinase                                           |
| gbs1734   | scrA        | -22.3| 2.6  |      | PTS system, sucrose-specific IIABC component           |
| gbs1735   | scrB        | -5.2|     |      | Sucrose-6-phosphate hydrolase                          |
| gbs1777   | glgF.2      | -   | 2.2  | 3.8  | Glycerol uptake facilitator protein                     |
| gbs1797   |             | -2.3|     |      | Galactose-1-phosphate uridylyltransferase              |
| gbs1811   | plr         | -   | 2.1  | 2.5  | Glyceraldehyde 3-phosphate dehydrogenase                |
| gbs1850   |             | -   | -2.5 | -9.9 | Transaldolase                                          |
| gbs1893   |             | -11.6| -6.5 |      | 2-dehydro-3-deoxygluconokinase                        |
| gbs1911   | dexB        | -   | 2.9  |      | Glucan 1,6-alpha-glucosidase                           |
| gbs1912   |             | -   | 2.9  |      | Multiple sugar transport ATP-binding protein msmK       |
| gbs1914   |             | -7.3| 169.1|      | Aldose 1-epimerase family protein                      |
| gbs1915   |             | -3.3| -61.2| -2.5 | Tagatose-bisphosphate aldolase                         |
| gbs1916   |             | -3.1| -92.2|      | Tagatose-6-phosphate kinase                            |
| gbs1917   |             | -3.1| 67.0 |      | Galactose-6-phosphate isomerase lacB subunit           |
| gbs1918   | lacA.1      | -4.2| 296.9|      | Galactose-6-phosphate isomerase lacA subunit           |
| gbs1919   |             | 14.8|     |      | Sialidase A precursor                                  |
| gbs1920   |             | 10.5|     |      | PTS system, galactose-specific IIC component           |
| gbs1921   |             | -2.3| 14.4 |      | PTS system, galactose-specific IIB component           |
| Locus   | Name       | ML  | LL  | S    | Putative function                              |
|---------|------------|-----|-----|------|------------------------------------------------|
| gbs1922 | -          | -3.6| -19.2|      | PTS system, galactose-specific IIA component   |
| gbs1923 | lacR.1     | -2.7| -3.4|      | Lactose phosphotransferase system repressor    |
| gbs1936 | ptsD       | -36.4| -10.1|      | PTS system, mannose fructose family IID component |
| gbs1937 | ptsC       | -45.8| -31.4|      | PTS system, mannose fructose family IIC component |
| gbs1938 | ptsB       | -37.6| -8.8 |      | PTS system, mannose fructose family IIB component |
| gbs1939 | -          | -11.1| -34.6| -42.6| PTS system, mannose fructose family IIA component |
| gbs1946 |            | 5.8 | 4.6 |      | PTS system, glucose-specific IIABC component   |
| gbs2116 | -          | -3.2| 2.3 |      | Glucose uptake family protein                  |

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Figure 5. Functional dynamics of transcriptional changes between gene expression in THY and AF. A. Bar graphs present the number of genes better expressed in THY or AF in three of the most prominently changed metabolic categories. B. Major metabolic trends in nutrient acquisition. Red symbolizes genes better expressed in AF, green better expressed in THY.
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