Introduction

Streptococcus pneumoniae is a Gram-positive bacterial pathogen in humans that has the ability to colonize the nasopharyngeal cavity of the nose [1]. In favorable environmental conditions it may have the ability to adapt to fluctuating levels of nutrients [5,6], such as the available carbon source. The expression of various carbon metabolic genes was altered, including a PTS operon (which we here denote as the bgu operon) that has high similarity with the cel locus. In contrast to the cel locus, the bgu operon is conserved in all sequenced strains of S. pneumoniae, indicating an important physiological function in the lifestyle of pneumococci. We next characterized the transcriptional regulation of the bgu operon in more detail. Its expression was increased in the presence of cellobiose, and decreased in the presence of glucose. A novel GntR-type transcriptional regulator (which we here denote as BguR) was shown to act as a transcriptional repressor of the bgu operon and its repressive effect was relieved in the presence of cellobiose. BguR-dependent repression was demonstrated to be mediated by a 20-bp DNA operator site (5'-AAAAATGTCTAGACAAATTT-3') present in PbguA, as verified by promoter truncation experiments. In conclusion, we have identified a new cellobiose-responsive PTS operon, together with its transcriptional regulator in S. pneumoniae.

Abstract

The human pathogen Streptococcus pneumoniae has the ability to use the carbon- and energy source cellobiose due to the presence of a cellobiose-utilizing gene cluster (cel locus) in its genome. This system is regulated by the cellobiose-dependent transcriptional activator CelR, which has been previously shown to contribute to pneumococcal virulence. To get a broader understanding of the response of S. pneumoniae to cellobiose, we compared the pneumococcal transcriptome during growth on glucose as the main carbon source to that with cellobiose as the main carbon source. The expression of various carbon metabolic genes was altered, including a PTS operon (which we here denote as the bgu operon) that has high similarity with the cel locus. In contrast to the cel locus, the bgu operon is conserved in all sequenced strains of S. pneumoniae, indicating an important physiological function in the lifestyle of pneumococci. We next characterized the transcriptional regulation of the bgu operon in more detail. Its expression was increased in the presence of cellobiose, and decreased in the presence of glucose. A novel GntR-type transcriptional regulator (which we here denote as BguR) was shown to act as a transcriptional repressor of the bgu operon and its repressive effect was relieved in the presence of cellobiose. BguR-dependent repression was demonstrated to be mediated by a 20-bp DNA operator site (5'-AAAAATGTCTAGACAAATTT-3') present in PbguA, as verified by promoter truncation experiments. In conclusion, we have identified a new cellobiose-responsive PTS operon, together with its transcriptional regulator in S. pneumoniae.
To get a broader understanding of the response of *S. pneumoniae* to cellobiose, we examined in this study cellobiose-dependent regulation on a transcriptome-wide level and found another operon, homologous to the cel locus, which is highly expressed in the presence of cellobiose. This operon encodes a PTS system that was recently shown to contribute to growth on the β-glucosides amygdalin and cellobiose [17]. In subsequent experiments, this operon was found to be regulated by a GntR-type transcriptional repressor (BguR, encoded by the divergently orientated upstream gene) in response to cellobiose and glucose. A conserved operator sequence was found that is necessary for the regulation to take place. The novel PTS operon, which we tentatively name *bguDBC*, did not contribute to growth in medium with cellobiose as the sole carbon source, whereas the cel locus did. Therefore, the cel locus seems to be the primary transport system for cellobiose in *S. pneumoniae* D39, but the exact function of the *bgu* operon, although likely to lie in transport of β-glucosides [17], is still not entirely clear.

**Materials and Methods**

**DNA Manipulation, Bacterial Strains and Growth Conditions**

Chromosomal DNA of *S. pneumoniae* D39 wild-type [10] was used for PCR amplification. Primers were based on the sequence of the D39 genome [10] and are listed in Table 1. Bacterial strains and plasmids used in this study are listed in Table 2. All bacterial strains were stored in 10% (v/v) glycerol at −80°C. M17 broth [22,23] supplemented with 0.5% (w/v) glucose was used for growing *S. pneumoniae* D39 wild-type [10] on blood agar plates supplemented with 1% (v/v) defibrinated sheep blood in micro-aerophilic conditions at 37°C. For selection, media were supplemented with the following concentrations of antibiotics: erythromycin: 0.25 μg ml⁻¹, spectinomycin: 150 μg ml⁻¹, tetracycline: 2.5 μg ml⁻¹ for *S. pneumoniae*, and ampicillin: 100 μg ml⁻¹ for *E. coli*.

**Construction of Deletion Mutants of *bguR* and *bguDBC***

*bguR* and *bguDBC* deletion mutants were made by allelic replacement with a spectinomycin- and erythromycin-resistance gene, respectively, following the procedure as described before [24]. Briefly, primer pairs SPD1830-KO-1/SPD1830-KO-2, SPD1830-KO-3/SPD1830-KO-4, 1832-4-KO-1/1832-4-KO-2 and 1832-4-KO-3/1832-4-KO-4 were used to generate PCR fragments of the left- and right flanking regions of *bguR* and *bguDBC*, respectively. The spectinomycin-resistance marker was amplified by a PCR on pORI38 with primers Spec-F/Spec-R. The erythromycin-resistance marker was amplified by a PCR on pORI28 with primers Ery-F/Ery-R. Then, the left- and right flanking regions of *bguR* and *bguDBC* were fused to the spectinomycin- and erythromycin-resistance markers, respectively, by means of overlap-extension PCR. The resulting PCR products were transformed to *S. pneumoniae* D39 wild-type and selection of the mutant strains was done with the appropriate antibiotic. Spectinomycin- and erythromycin-resistant clones were further examined for the presence of the *bguR* and *bguDBC* deletion, respectively, by PCR.

### Table 1. List of primers used in this study. Restriction sites are underlined.

| Name       | Nucleotide Sequence (5’ 3’)              | Restriction site |
|------------|----------------------------------------|-----------------|
| PbguA-Fr   | CGGATCCCGCTAGAAGCTGCTCCCCACC           | EcoRI           |
| PbguA-Rv   | CGGAATTCCTTTTACGAATCTCATTGT            | BamHI           |
| PmalQ-Fr   | CGGGAATCTATGAGGACCTTGTGCTCTTCACC      | EcoRI           |
| PmalQ-Rv   | CGGATCCGAGATGTGCATCAACACAC             | BamHI           |
| PmalP-Fr   | CGGGAATCCTCTCTAGAGAATC                | EcoRI           |
| PmalP-Rv   | CGGAATCCAGCACCGCAATGCTC               | BamHI           |
| SPD1830-KO-1 | GTAAATTCATCACAAGATCC                   |                |
| SPD1830-KO-2 | TCTCCCTCAATATTGATGTGGCTATTAACTTGG    |                |
| SPD1830-KO-3 | CTGGTTCAGCTTTCTGATCAGCAGCTCCTTGG      |                |
| SPD1830-KO-4 | CTGTTTTCATCATTTTCCC                  |                |
| SPD1832-4-KO-1 | CTGGATGCGACAGATAC                   |                |
| SPD1832-4-KO-2 | GAGAATCTATCAGATGCGACGACGAGGCAATTGG  |                |
| SPD1832-4-KO-3 | AGTATGCGCTTATGTTAATCTCAGTCATCTATC   |                |
| SPD1832-4-KO-4 | TCTCTGATGAGTTGTCAC                 |                |
| Spec-R     | ACTAACAGAAATTAACGC                   |                |
| Spec-F     | CTATCACAATAGTGAGGAGG                 |                |
| Ery-R      | TAAAGATTAGCCGATAACT                 |                |
| Ery-F      | GATACATGCAATGATCCTCTG                |                |

**Forward primers used with PbguA-Rv (reverse primer) for 5’ subcloning of PbguA**

| PbguA-5.3 | GCCGAATCTTGAACTTGGCAATTTTTTTTTAAT      | EcoRI           |
| PbguA-5.4 | GCCGAATCTTGAACTTGGCAATTTTTTTTATAGC    | EcoRI           |
| PbguA-5.5 | GCCGAATCTTGAACTTGGCAATTTTTTTTTAAT     | EcoRI           |

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**Table 2.** List of strains and plasmids used in this study.

| Strain/plasmid | Description | Source |
|----------------|-------------|--------|
| **S. pneumoniae** | | |
| D39 | Serotype 2 strain, cps 2 | Laboratory of P. Hermans. |
| ΔcfaA | D39 ΔcfaA, Spec<sup>e</sup> | [13] |
| ΔcelR | D39 ΔcelR, Spec<sup>e</sup> | [8] |
| ΔbguRBC | D39 ΔbguRBC, Ery<sup>R</sup> | This study |
| SS300 | D39 ΔbguR, Spec<sup>e</sup> | This study |
| SS301 | D39 ΔbguA-PbguA-lacZ, Tet<sup>R</sup> | This study |
| SS302 | SS300 ΔbguA-PbguA-lacZ, Tet<sup>R</sup> | This study |
| SS303 | ΔcfaA ΔbguA-PbguA-lacZ, Tet<sup>R</sup> | This study |
| SS304 | ΔbguRBC ΔbguA-PbguA-lacZ, Tet<sup>R</sup> | This study |
| SS305 | D39 ΔbguA-PbguA-5.3-lacZ, Tet<sup>R</sup> | This study |
| SS306 | D39 ΔbguA-PbguA-5.4-lacZ, Tet<sup>R</sup> | This study |
| SS307 | D39 ΔbguA-PbguA-5.5-lacZ, Tet<sup>R</sup> | This study |
| **E. coli** | | |
| EC1000 | Km<sup>R</sup>; MC1000 derivative carrying a single copy of the pWV1 repA gene in glgB | Laboratory collection |
| **Plasmids** | | |
| pPP2 | Amp<sup>R</sup>; Tet<sup>R</sup>; promoter-less lacZ. For replacement of bgaA with promoter lacZ-fusion. Derivative of pTP1. | [25] |
| pSS301 | pPP2 Pbgua-lacZ | This study |
| pSS302 | pPP2 Pbgua-5.3-lacZ | This study |
| pSS303 | pPP2 Pbgua-5.4-lacZ | This study |
| pSS304 | pPP2 Pbgua-5.5-lacZ | This study |

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Construction of lacZ-fusions and bguA Promoter Subclones in pPP2

The pPP2 [25] plasmid was used to construct a vector for an ectopic chromosomal transcriptional lacZ-fusion to the promoter of bgaA in S. pneumoniae D39 using the primer pair mentioned in Table 1. The resulting plasmid pSS301 was introduced into D39 wild-type and the bguR mutant and integrated in the genome via double crossover in the bgaA gene, resulting in strains SS301 and SS302. The Pbgua-<sup>+</sup> was also introduced into the ΔcfaA strain that was published before [13] and in the ΔbguRBC, resulting in strain SS303 and SS304, respectively.

The following promoter subclones of Pbgua were made in pPP2 (primer pairs are mentioned in Table 1): Pbgua<sup>-5.3</sup> (truncated 15 bases upstream of the proposed BguR operator site), Pbgua<sup>-5.4</sup> (first 6 bases of the BguR operator site deleted) and Pbgua<sup>-5.5</sup> (half of the BguR operator site deleted, but keeping the -35 site intact). This resulted in plasmids pSS302-04. These constructs were introduced into D39 wild-type as described above, resulting in strains SS303-07. All plasmid constructs were checked by sequencing.

Enzyme Assays

Specific β-galactosidase assays were performed as described before [26]. Cells were grown in M17 in the presence of the appropriate carbon source (exact concentrations are mentioned in the Results section) and harvested in the mid-exponential phase of growth.

DNA Microarray Analyses

For transcriptome analyses of S. pneumoniae, the D39 wild-type strain was grown in 3 biological replicates in CM17 (0.5% Cellbiose+M17) and compared to the strain grown in GM17 (0.5% Glucose+M17) medium. Cells were harvested for RNA isolation at two time points in CM17 (CT-1 and CT2) and GM17 (GT-1 and GT-2) medium (see Fig. 1). To analyze the effect of bguR deletion on the transcriptome, S. pneumoniae D39 wild-type and its isogenic bguR mutant (SS300) were grown in 3 biological replicates in GM17 (0.5% Glucose+M17) medium. These cultures were harvested at the mid-exponential phase of growth at an OD600 of 0.25. All other procedures regarding the DNA microarray experiments (cell disruption, RNA isolation, RNA quality testing, cDNA synthesis, labeling with dyes (Cy3 and Cy5), hybridization and scanning) were performed as described before [27,28].

DNA Microarray Data Analysis

DNA microarray data were analyzed as described before [27–29]. For identification of differentially expressed genes a Bayesian p-value <0.001 and fold change cut-off of 3 was applied. Microarray data have been submitted to GEO under accession number GSE43345.

In silico Analyses

The NCBI web site (www.ncbi.nlm.nih.gov/BLAST/) was used for blasting genes among different genomes, while the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/) database was used to analyze different genomes of S. pneumoniae (D39, Tigr4, R6, GSSP14, G54-19F, ATCC700669, Hungary-
of cellobiose, cells have to adapt in the first phase and are therefore functional category J (translation, ribosomal structure and T1 and T2). At T1 genes that were significantly differentially expressed at time points corresponding proteins (see table S1 for expression ratios of all categories on the basis of the putative functions of the affected genes in these transcriptome profiling experiments, indicated in Fig. 1. Table 3 summarizes the number of genes that were affected in these transcriptome profiling experiments, with a high number of differentially expressed genes are S (function unknown) and R (general functions prediction only).

The effects of cellobiose on the transcriptome of S. pneumoniae D39 that were observed at both time points are summarized in Table 4. The expression of various genes and operons of diverse function was altered in the presence of cellobiose instead of glucose as the added carbon source, among which sugar metabolic genes. Notably, genes involved in cellobiose and maltose metabolism were highly upregulated. In addition, an ABC transporter cluster (msmEFG) that encodes a putative multiple sugar transport system was highly upregulated as well. Cellobiose metabolism has previously been shown to be carried out by a cellobiose-specific gene cluster (cel locus) in streptococci, including S. pneumoniae [7,8,21]. In a previous study, we showed that CelR is involved in the activation of the cel locus, specifically in the presence of cellobiose [8]. As expected, the cel locus was also among the highly upregulated genes at both time points. This suggests that the conditions applied for the transcriptome analyses are indeed appropriate to find cellobiose-responsive genes. The expression of an operon (SPD1830–1833, which will be denoted here tentatively as the bgu operon) encoding a glycosyl hydrolase protein and the PTS system IICBA components was also highly upregulated at both time points of growth. Interestingly, blast searches showed that this operon has high similarity with the cel locus that was previously identified to be involved in cellobiose metabolism [7,8]. In addition, this operon was recently shown to play a role in the metabolism of the β-glucosides cellobiose and amygdalin [17]. Our transcriptome data support a role of this operon in the metabolism/utilization of cellobiose or similar carbon sources. Therefore, we decided to further investigate the regulation of this operon in the presence of cellobiose.

Results

Growth and Transcriptome Analysis of S. pneumoniae in Medium with Cellobiose as the Sole Carbon Source

To study the growth behavior of S. pneumoniae D39 in cellobiose, growth experiments were performed in the complex M17 medium supplemented with 0.5% cellobiose, which was compared to the growth in M17+0.5% glucose. As expected, normal exponential growth of D39 was observed in the presence of glucose. However, on cellobiose cells grew in a diauxic growth pattern with two distinct growth phases. This is similar to the growth behavior of S. pneumoniae on β-glucosides like cellobiose as seen in a recent study [18]. As the first exponential growth phase is similar to the growth in M17 without added carbon source (Fig. 1), it is most likely that cellobiose is only metabolized in the second growth phase. Because of these two growth phases on cellobiose, we decided to perform a transcriptome comparison with the growth on glucose in both phases (T1 and T2, Fig. 1) and in this way identify cellobiose-responsive genes. Therefore, D39 wild-type was grown in CM17 (0.5% Cellobiose+M17) and in GM17 (0.5% Glucose+M17) and cells were harvested at two different time points (T1 and T2) as indicated in Fig. 1. Table 3 summarizes the number of genes that were affected in these transcriptome profiling experiments, grouped into COG (Clusters of Orthologous Groups) functional categories on the basis of the putative functions of the corresponding proteins (see table S1 for expression ratios of all genes that were significantly differentially expressed at time points T1 and T2). At T1 ~10 times more genes were affected in the presence of cellobiose than at T2, comprising many genes of COG functional category J (translation, ribosomal structure and biogenesis). This probably reflects the fact that in the presence of cellobiose, cells have to adapt in the first phase and are therefore not growing optimally, whereas in the second phase the cellobiose is likely to be actively metabolized. At both time points (T1 and T2), most of the affected genes belong to COG functional category G (carbohydrate transport and metabolism). Almost all the affected genes belonging to category G (49 out of 51 at T1 and 16 out of 20 at T2) were upregulated in the presence of cellobiose. These effects may be either due to release of carbon catabolite repression [13] of these genes as an effect of the absence of glucose, or due to a direct inductive effect of cellobiose. Other COG functional categories with a high number of differentially expressed genes are S (function unknown) and R (general functions prediction only).

Figure 1. Growth of S. pneumoniae D39 in the presence of 0.5% cellobiose (grey line ■) and 0.5% glucose (black line ▲) in M17 medium. Black circles show the time points at which cultures were harvested for transcriptome analysis. Where C = cellobiose, G = Glucose, T1 = time point 1 and T2 = Time point 2. doi:10.1371/journal.pone.0057586.g001

Organization and Conservation of the bgu Operon in S. pneumoniae

The bgu locus spans the genes SPD1830–33 (Fig. 2). SPD1830 (here named bguA) encodes a glycosyl hydrolase belonging to the BglB family, which has high similarity (30% identity) to celA of S. pneumoniae and other streptococci. Next, the downstream three genes (SPD1831–33, named bguDBC), encode PTS system IICBA components that show high similarity (29–30% identity) with celDCEB located in the cel locus of various streptococci, including S. pneumoniae. Upstream of bguA, a gene encoding a GntR family transcriptional factor (named bguR) is located. The presence of this transcription factor in the DNA region upstream of the bgu operon indicates that it may function as a transcriptional regulator of the bgu operon (see also below).

In a previous study, we have studied the role of CelR in the regulation of the cel locus [8]. However, the cel locus was found to be absent from 50% of the S. pneumoniae strains present in the KEGG database. We also analyzed all the strains of S. pneumoniae available on the KEGG website for the presence of the bgu operon. Notably, this operon is 100% conserved in all the sequenced strains of S. pneumoniae available on KEGG database. This suggests an important role of this operon in carbohydrate metabolism,
expression of P
concentration of glucose, with an increasing concentration of
overrule the first when present at the same time.
decreases its expression, whereby the last compound appears to
bguA
Cellobiose increases the expression of P
not be increased by adding 0.5% cellobiose to the medium. Thus,
SPD1829
(Cyber-T test).
To further investigate the role of glucose in the regulation of
bgu Operon
To examine whether the observed altered expression of the bgu operon in the transcriptome analyses was due to a specific effect of cellobiose, we constructed an ectopic transcriptional lacZ-fusion to the bguA promoter in the D39 wild-type strain. This strain was grown in M17 medium supplemented with 0.5% of different carbon sources (Cellobiose, Fructose, Galactose, Glucose, Lactose, Mannose, Maltose, NAG, Raffinose, Sucrose and Trehalose). The highest expression of P_{bguA} was observed in the presence of cellobiose (Table 5). The presence of glucose led to the lowest expression of P_{bguA}. These results confirm the microarray data and additionally show that expression of P_{bguA} is controlled specifically by cellobiose.

To further investigate the role of glucose in the regulation of P_{bguA}, we grew the cells in the presence of a certain constant concentration of glucose, with an increasing concentration of cellobiose, as mentioned in Table 6. At 0.2% glucose, the lowest expression of P_{bguA} was observed. This level of expression could not be increased by adding 0.5% cellobiose to the medium. Thus, cellobiose increases the expression of P_{bguA}, whereas glucose decreases its expression, whereby the last compound appears to overrule the first when present at the same time.

**SPD1829 (bguR) is a Repressor of the bgu Operon**

Adjacent to the bgu operon, a putative GntR-family regulator is located (SPD1829), which we named BguR. We hypothesized that this regulator could be involved in the observed regulation of the bgu operon. To investigate the role of BguR, the bguR gene was replaced by a spectinomycin-resistance marker by means of allelic replacement. To examine the effect of the bguR deletion on the transcriptome of *S. pneumoniae*, the transcriptome of the bguR mutant strain (SS300) was compared to that of the D39 wild-type strain grown in GM17 (0.5% glucose+M17) medium. GM17 medium was used, since low expression of the bgu operon was observed in the presence of glucose, which we hypothesized to represent a condition with maximal repression of the bgu operon by BguR. These transcriptome data revealed that the expression of 13 genes was significantly altered due the deletion of bguR (Table 7). The expression of bguR was five-fold downregulated confirming the inactivation of the bguR gene in the bguR deletion strain (SS300). The most highly upregulated genes were the ones constituting the bgu operon (more than 20-fold), indicating a role/function of BguR as the repressor of the bgu operon. Another strong effect caused by deletion of bguR was the upregulation of an operon involved in maltose/maltodextrin metabolism [30]. These results show that inactivation of bguR brings about only a modest change in the transcriptome of *S. pneumoniae* D39, and furthermore imply a role of BguR in repressing the expression of the bgu operon.

**Regulation of P_{bguA} in ccaA, bguR and bguDBC Mutant Strains**

To further confirm that BguR is responsible for the repression of the bgu operon that was upregulated in the bguR mutant strain (SS300), we introduced the P_{bguA}-lacZ into D39 wild-type and the bguR mutant. In GM17 (0.5% Glucose+M17) medium specific β-

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**Table 3. Number of genes significantly* affected in the presence of cellobiose at time point T1 and T2.**

| Functional Categories | T1 | T2 |
|-----------------------|----|----|
| Total Down Up         |    |    |
| C: Energy production and conversion | 7 1 6 | 1 0 1 |
| D: Cell cycle control, cell division, chromosome partitioning | 5 4 1 | 0 0 0 |
| E: Amino acid transport and metabolism | 18 16 2 | 0 0 0 |
| F: Nucleotide transport and metabolism | 14 12 2 | 0 0 0 |
| G: Carbohydrate transport and metabolism | 52 2 50 | 9 0 9 |
| H: Coenzyme transport and metabolism | 9 4 5 | 0 0 0 |
| I: Lipid transport and metabolism | 3 2 1 | 0 0 0 |
| J: Translation, ribosomal structure and biogenesis | 49 46 3 | 0 0 0 |
| K: Transcription | 12 7 5 | 1 0 1 |
| L: Replication, recombination and repair | 13 10 3 | 0 0 0 |
| M: Cell wall/membrane/envelope biogenesis | 15 9 6 | 0 0 0 |
| Q: Secondary metabolites biosynthesis, transport and catabolism | 1 0 1 | 0 0 0 |
| R: General function prediction only | 27 13 14 | 2 0 2 |
| S: Function unknown | 51 22 29 | 10 2 8 |
| T: Signal transduction mechanisms | 3 1 2 | 0 0 0 |
| U: Intracellular trafficking, secretion, and vesicular transport | 2 1 1 | 0 0 0 |
| V: Defense mechanisms | 12 3 9 | 0 0 0 |
| Total number of genes | 307 163 144 26 2 24 |

*Representing the genes with at least 3-fold increase or 3-fold decrease in expression levels in CM17 compared to GM17, and with a Bayesian p-value below 0.001 (Cyber-T test).
doi:10.1371/journal.pone.0057586.t003
galactosidase activity was highly increased in the bguR mutant as compared to D39 wild-type (Table 8). This not only confirms the data of the bguR mutant transcriptome but also shows that BguR carries out repression of the bgu operon via the PbguA.

CcpA is considered a master transcriptional regulator in the control of carbohydrate utilization and metabolism genes in Gram-positive bacteria including S. pneumoniae [13,31,32]. To investigate a possible role of CcpA in the regulation of the bgu operon, we measured specific β-galactosidase activity of PbguA-lacZ in a ccpA mutant strain. No difference in expression of PbguA was observed in the ccpA mutant as compared to the wild-type strain when cells were grown in M17 with either cellobiose or glucose.

| D39 locus tag | Function (TIGR Annotation) | Ratio |
|--------------|-----------------------------|-------|
| SPD0265      | Alcohol dehydrogenase, zinc-containing | 26.0 2.1 |
| SPD0277      | 6-Phospho-beta-glucosidase, CelA | 71.9 88.4 |
| SPD0278      | Hypothetical protein | 2.1 3.5 |
| SPD0279      | Cellobiose phosphotransferase system IIIB component, CelB | 13.9 23.4 |
| SPD0280      | DNA binding transcriptional regulator, CelR | 10.3 10.5 |
| SPD0281      | Cellobiose phosphotransferase system IIA component, CelC | 11.7 6.9 |
| SPD0282      | Hypothetical protein | 12.8 4.3 |
| SPD0283      | Cellobiose phosphotransferase system IIC component, CelD | 8.8 3.7 |
| SPD0344      | DNA-binding response regulator | 3.5 2.8 |
| SPD0466      | BlpT protein fusion | 4.3 4.0 |
| SPD0473      | Immunity protein BlpY | 11.8 5.6 |
| SPD0502      | PTS system, beta-glucosides-specific IABC components | 10.1 1.3 |
| SPD0503      | 6-phospho-beta-glucosidase | 14.6 1.1 |
| SPD0661      | PTS system IABC components | 2.0 2.1 |
| SPD0850      | Lactoylglutathione lyase, GloA | −20.6 2.5 |
| SPD0851      | Dihydroorotate dehydrogenase, PyrK | −150.3 2.8 |
| SPD0852      | Dihydroorotate dehydrogenase 1B, PyrDb | −65.8 2.3 |
| SPD0886      | Thioredoxin family protein | 12.6 3.6 |
| SPD1495      | Sugar ABC transporter, sugar-binding protein | 8.5 7.1 |
| SPD1496      | PTS system, IIBC components | 2.7 2.7 |
| SPD1590      | General stress protein 24, putative | 5.5 5.1 |
| SPD1675      | Sugar ABC transporter, MsmG | 8.2 13 |
| SPD1676      | Sugar ABC transporter, MsmF | 11.9 1.7 |
| SPD1677      | Sugar ABC transporter, MsmE | 10.7 2.1 |
| SPD1726      | Pneumolysin, PIY | −2.0 −2.0 |
| SPD1727      | Hypothetical protein | −2.7 −2.5 |
| SPD1728      | Hypothetical protein | −3.5 −3.6 |
| SPD1729      | Hypothetical protein | −8.64 −3.4 |
| SPD1830      | Glycosyl hydrolase family 1, BguA | 57.1 2.3 |
| SPD1831      | PTS system, IIC component, BguD | 62.9 3.4 |
| SPD1832      | PTS system, IIB component, BguB | 53.4 4.2 |
| SPD1833      | PTS system, IIA component, BguC | 55.9 3.3 |
| SPD1865      | Alcohol dehydrogenase, Zinc-containing | 12.6 2.5 |
| SPD1866      | N-Acetylglucosamine-6-phosphate deacetylase, NagA | 4.2 2.5 |
| SPD1933      | Glycogen phosphorylase family protein | 1.3 1.7 |
| SPD1934      | 4-Alpha-glucanotransferase, MalQ | 2.1 3.4 |
| SPD1935      | Maltose/maltodextrin ABC transporter, MalX | 1.3 2.1 |
| SPD1936      | Maltodextrin ABC transporter, MalC | 1.6 1.8 |

*Gene numbers refer to D39 locus tags.

†D39/TIGR4 annotation [10,15,47].

*Ratio represents the fold increase in the expression of genes in CM17 as compared to GM17. In some cases neighbouring genes with lower than 3-fold ratios are also indicated.

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Table 4. List of genes that are differentially expressed in the transcriptome comparison of S. pneumoniae D39 strain grown in CM17 and GM17 at time points T1 and T2.
(Table 8). In addition, no CcpA binding site (cre) was found in the PbguA promoter [13]. Thus, these data show that regulation of the bgu operon in S. pneumoniae is independent of CcpA.

In S. mutans, regulation of the cel locus by CelR requires the phosphorylation of CelR by one of the PTS components, namely CelD [21]. Therefore, to investigate a possible role of the BguDBC proteins in the regulation of the bgu operon via an effect on BguR activity, we measured specific β-galactosidase activity of PbguA-lacZ in a bguDBC mutant strain. No difference in expression of PbguA was observed in the bguDBC mutant as compared to the wild-type D39 strain, when cells were grown in M17 medium with cellobiose or glucose (Table 8). This suggests that the components of the Bgu PTS system are not required for activation of BguR in the presence of cellobiose. However, we cannot exclude that other PTS systems or signaling cascades confer a regulatory effect on BguR.

Identification of the BguR Operator Site

The data presented above strongly suggest a direct effect of BguR on PbguA. To identify a possible BguR operator sequence, a 5’ promoter truncation study was performed with the PbguA promoter. A diagram of the PbguA promoter truncation is shown in Fig. 3. Truncation of PbguA near to the predicted -35 core promoter sequence (PbguA-5.4) relieved the repressive action of BguR on PbguA, suggesting the presence of a putative BguR operator in this deleted region of the promoter. Further bioinformatics analysis of this area revealed the presence of a

| Specific β-galactosidase activity (Miller units) in M17 medium | PbguA-lacZ |
|------------------|-------------|
| No               | 124 (4)     |
| Cellobiose       | 515 (6)     |
| Fructose         | 121 (9)     |
| Galactose        | 122 (7)     |
| Glucose          | 105 (6)     |
| Lactose          | 125 (9)     |
| Mannose          | 129 (5)     |
| Maltose          | 131 (6)     |
| NAG              | 130 (8)     |
| Rafinose         | 116 (7)     |
| Sucrose          | 103 (5)     |
| Trehalose        | 125 (4)     |

Standard deviation of 3 independent experiments is given in parentheses.
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Table 6. Specific β-galactosidase activity (miller units) of D39 wild-type containing the PbguA-lacZ fusion grown in M17 medium with different combinations of added sugars (% w/v).

| Sugars       | PbguA-lacZ |
|--------------|------------|
| No           | 115 (2)    |
| 0.1% C       | 205 (3)    |
| 0.2% C       | 340 (5)    |
| 0.3% C       | 380 (5)    |
| 0.5% C       | 490 (17)   |
| 1% C         | 650 (13)   |
| 0.1% G       | 109 (2)    |
| 0.2% G       | 107 (3)    |
| 0.3% G       | 103 (7)    |
| 0.5% G       | 105 (5)    |
| 1% G         | 101 (8)    |
| 0.1% G +0.1% C | 112 (6)   |
| 0.1% G +0.2% C | 122 (4)   |
| 0.1% G +0.5% C | 140 (8)   |
| 0.2% G +0.1% C | 109 (1)   |
| 0.2% G +0.2% C | 108 (3)   |
| 0.2% G +0.5% C | 112 (5)   |

Standard deviation of 3 independent experiments is given in parentheses. G, Glucose. C, Cellobiose.
doi:10.1371/journal.pone.0057586.t006

| Specific β-Galactosidase Activity (Miller Units) in M17 medium | PbguA-lacZ |
|------------------|-------------|
| No               | 115 (2)     |
| 0.1% C           | 205 (3)     |
| 0.2% C           | 340 (5)     |
| 0.3% C           | 380 (5)     |
| 0.5% C           | 490 (17)    |
| 1% C             | 650 (13)    |
| 0.1% G           | 109 (2)     |
| 0.2% G           | 107 (3)     |
| 0.3% G           | 103 (7)     |
| 0.5% G           | 105 (5)     |
| 1% G             | 101 (8)     |
| 0.1% G +0.1% C   | 112 (6)     |
| 0.1% G +0.2% C   | 122 (4)     |
| 0.1% G +0.5% C   | 140 (8)     |
| 0.2% G +0.1% C   | 109 (1)     |
| 0.2% G +0.2% C   | 108 (3)     |
| 0.2% G +0.5% C   | 112 (5)     |

Standard deviation of 3 independent experiments is given in parentheses. G, Glucose. C, Cellobiose.
doi:10.1371/journal.pone.0057586.t006

Table 7. Summary of transcriptome comparison of S. pneumoniae strain D39 ΔbguR and D39 wild-type grown in GM17.

| D39 locus taga | Functionb | Ratioc |
|---------------|-----------|--------|
| SPD0311       | Glucan 1,6-alpha-glucosidase, DexB | 3.2 |
| SPD0771       | Lactose phosphotransferase system repressor, LacR | 4.3 |
| SPD0772       | 1-phosphofructokinase, putative | 4.1 |
| SPD0773       | PTS system, fructose specific IIABC components | 4.0 |
| SPD1830       | Glycosyl hydrolase, family 1, BguA | 89.6 |
| SPD1831       | PTS system, IIC component, BguD | 127.8 |
| SPD1832       | PTS system, IIIB component, BguB | 47.4 |
| SPD1833       | PTS system, IIAB component, BguC | 21.8 |
| SPD1932       | Glycogen phosphorylase family protein | 10.0 |
| SPD1933       | 4-alpha-glucanotransferase, MalQ | 14.0 |
| SPD1934       | Maltose/maltodextrin ABC transporter, MalX | 4.7 |
| SPD1935       | Maltodextrin ABC transporter, MalC | 2.6 |
| SPD1829       | GntR family transcriptional regulator, BguR | −5.2 |

aGene numbers refer to D39 locus tags. 
bD39 annotation/TIGR4 annotation [10,15,47].
cRatio represents the fold increase in the expression of genes in CM17 as compared to GM17.
doi:10.1371/journal.pone.0057586.t007
20-bp palindromic region (5'-AAAAATGTCTAGACAATTTTT-3') that is overlapping with the -35 site and that might act as the BguR operator site. Deletion of half of this predicted operator site (PbguA-5.5) led to high expression of PbguA in CM17 (0.5% Cellobiose+M17) and GM17 (0.5% Glucose+M17) medium in the wild-type (Fig. 3). However, when the PbguA was truncated only a few base pairs upstream of the predicted operator site (PbguA-5.3), expression was similar to that of the full-length promoter (Fig. 3). Therefore, these data suggest that the predicted operator site is functional and acts as the BguR operator site.

The entire genome of S. pneumoniae D39 was searched for the presence of the BguR operator site by using Genome2D. Also, the promoters of the putative fructose and maltose utilization gene clusters were analyzed manually for the presence of the BguR operator site. However, we could not find a sequence that resembles it, suggesting that the bgu operon is the only direct target of BguR.

The cel Locus Is Required for D39 to Grow on Cellobiose, While the bgu Operon is Not

As in a previous study it had been shown that deletion of celR or celD from the cel locus leads to growth inhibition in CDM with cellbiose the carbon source [7], and since the bgu locus was implicated in the uptake of cellbiose and amygdalin [17], we decided to mutate bguDBC and compare the growth of ΔbguDBC, ΔcelR (which has no expression of the cel locus) [7,8], and ΔcelR-bguDBC in the presence of 0.5% cellbiose and 0.5% glucose with that of D39 wild-type (Fig. 4). In the presence of glucose, all strains grew similar as the wild-type. However, clear growth differences were observed in the presence of cellbiose. As mentioned before, D39 wild-type grows with two distinct exponential phases in the presence of cellbiose. A similar growth pattern was observed for the ΔbguDBC mutant strain, but the ΔcelR and ΔcelR-bguDBC mutant strains were not able to start the second exponential phase. These data suggest that the cel locus is important for S. pneumoniae D39 to grow on cellbiose, while under these conditions the bgu operon, although its expression is responsive to cellbiose, is not.

**Table 8.** Specific β-Galactosidase activity (miller units) of D39 wild-type, ΔbguR, ΔcelA, and ΔbguDBC mutants all containing the PbguA-lacZ transcriptional fusion grown in M17 medium supplemented with added concentrations (0.5% w/v) of cellbiose (C) and glucose (G).

| Locus (strain) | WT | ΔbguR | ΔcelA | ΔbguDBC |
|---------------|----|-------|-------|---------|
| PbguA (GM17)  | 115 (5) | 1660 (17) | 111 (8) | 116 (9) |
| PbguA (CM17)  | 610 (10) | 1690 (15) | 580 (12) | 599 (17) |

Standard deviation of three independent measurements is given in parentheses. The oval indicates the position of the putative BguR operator site, while the sequence of the BguR operator site is given above.

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**Figure 3.** Analysis of truncations of PbguA. A schematic overview of the bguA promoter truncations is shown. The table on the right gives the specific β-galactosidase activity of the truncated promoters in GM17 (0.5% glucose+M17) and CM17 (0.5% cellbiose+M17). Standard deviation is given in parentheses. The oval indicates the position of the putative BguR operator site, while the sequence of the BguR operator site is given above.

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**Discussion**

In many bacteria, including S. pneumoniae, glucose is considered one of the primary sources of energy for metabolic processes. However, the existence of numerous other sugar-specific systems in S. pneumoniae implies its ability to utilize various other carbon sources in the absence of glucose [10,15–17]. The role of different systems dedicated to the uptake and metabolism of different sugars including cellbiose, maltose, galactose, sucrose and raffinose has been explicitly investigated for S. pneumoniae [7,8,11,13,16,17,30,33–36]. In a previous study, we described the role of the cellbiose-dependent transcriptional activator (CelR) in the regulation of the cellbiose utilization gene cluster (cel locus) [8]. Intriguingly, the cel locus is not conserved in all the sequenced strains of S. pneumoniae that are available on the KEGG website. This might indicate that S. pneumoniae harbors other ways of cellbiose utilization. To be able to more thoroughly understand the effect of cellbiose on S. pneumoniae, we applied transcriptome profiling and various ways to study transcriptional gene regulation. The data show that, besides the cel locus, expression of a second operon (bgu) is responsive to cellbiose as well, which we show to be mediated by the transcriptional regulator BguR. The fact that the bgu operon is 100% conserved in...
M17 (A), 0.5% glucose isogenic mutants celR physiological function in the lifestyle of different pneumococcal strains suggests that it has an important role during growth on glucose to that on cellobiose and other β-glucosides. In addition, PTS SPD0502, which might transport β-glucosides as well [18], was found to be responsible for the extended lag phase, whereas the Bgu PTS that we describe here did not. Interestingly, the gene encoding the SPD0502 PTS was found to be upregulated during growth on cellobiose at T1 in our microarray experiment described in this study (Table 4 and S1). Thus, complex regulatory interactions around CelR take place.

In the BguR amino acid sequence no similar regulatory domains could be identified. In agreement with this, we could not find an effect of deletion of the bgu PTS genes on the cellobiose- and glucose-dependent regulation of PgmuA via BguR. Therefore, it remains to be determined how the signal of the carbohydrate source is transferred to BguR. In addition, no effect of CcpA on the regulation of the bgu operon could be detected, in accordance with previous studies [13]. Thus, expression of the bgu locus seems to be governed by a single transcriptional regulator, BguR, possible via direct binding of glucose and cellobiose to its C-terminal UTRA (UbiC transcription regulator-associated) domain and the regulation of the bgu locus is independent of the Cel system.

As reported in our previous study, the cel locus and the CelR regulatory site of S. pneumoniae were found to be highly conserved in other streptococci, although not all pneumococcal strains contain this operon [7]. Blast searches revealed a high conservation of the bgu operon in all the strains of S. pneumoniae that are available on the KEGG website. However, no similar bgu operon organization could be found in other streptococcal species using BLAST searches. Thus, the bgu operon seems to be specific for S. pneumoniae.

Blast searches with BguR revealed high similarity of this protein with the previously characterized GntR-type transcriptional regulator GmuR in Bacillus subtilis [41], BgcR in Escherichia coli [42] and DasR in Streptomyces coelicolor [43]. Moreover, the BguR operator site that we proposed in this study is also similar to the predicted GmuR (5’-tAAATGTaTAGACAttTa-3’) operator site in the order of bacillales [44]. GmuR was found to be involved in the regulation of the gmuBACDREFG operon, encoding glucosamnan utilization genes [41]. Expression of this operon was induced by cellobiose and mannobiose, which are possible degradation products of the action of GmuG on glucosamnan, and repressed by glucose [41]. Interestingly, GmuBAC has 31–46% identity with BguDBC while GmuR has 46% identity with BguR. Although we did not observe an effect of deletion of the bgu operon on growth on cellobiose, it could be involved in the utilization of other glucosamnan degradation products such as 4-O-β-D-mannopyranosyl-β-D-mannopyranose, 4-O-β-D-glucopyranosyl-β-D-mannopyranose. Intestinal anaerobic bacteria encounter glucosamnan and are able to degrade and ferment it [45]. Therefore, it is possible that the degradation products of glucosamnan are present in the environment of S. pneumoniae as well, by uptake from the intestine.

Figure 4. Growth of S. pneumoniae D39 wild-type (●) and its isogenic mutants celR (■), bguDBC (▲) and celR-bguDBC (X) in M17 (A), 0.5% glucose+M17 (B) and 0.5% cellobiose+M17 (C). doi:10.1371/journal.pone.0057586.g004

different pneumococcal strains suggests that it has an important physiological function in the life style of S. pneumoniae. Indeed, like celR, bguD was also found in one of the STM (Signature-tagged mutagenesis) studies, where it was implicated in lung infection [37].

The phosphoenolpyruvate-dependent (PEP) phosphotransferase system (PTS) is a major carbohydrate uptake system in bacteria, which not only phosphorylates different carbohydrates during uptake but also plays a major role in genetic regulation of metabolic activities [38–40]. In S. mutans, it has been shown that CelR has two PRD (PTS regulation domain) domains and phosphorylation (by glucose and cellobiose PTSs) at H226, H332 and H576 is required for the activation of CelR in the presence of cellobiose, while phosphorylation of H284 and H391 leads to inhibition of the CelR-dependent activation in the presence of glucose [21]. A similar role of these histidine residues as well as a phosphorylatable cysteine residue located in the EIIB domain of CelR is also elaborated on in S. pneumoniae [18]. The phosphorylatable cysteine was shown to affect the length of the lag phase during the shift from growth on glucose to that on cellobiose and other β-glucosides. In addition, PTS SPD0502, which might transport β-glucosides as well [18], was found to be responsible for the extended lag phase, whereas the Bgu PTS that we describe here did not. Interestingly, the gene encoding the SPD0502 PTS was found to be upregulated during growth on cellobiose at T1 in our microarray experiment described in this study (Table 4 and S1). Thus, complex regulatory interactions around CelR take place.

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BguR is a positive transcriptional regulator of a bgu operon which consists of 5 genes including encoding PTS subunits IIB (BgcE), IIA (BgcF), IIC (BgcI) and phospho-β-glucosidase (BgcA), and is found to be involved in the utilization of cellobiose and other β-glucosides (arbutin and salicin) at low temperature in E. coli [42]. However, salicin was not found to be fermented by the Bgc system and arbutin was shown to be taken up by the Cel system in S. pneumoniae [7,17]. Given the high similarity of the Bgc proteins with the proteins encoded in the bgu operon, we cannot exclude a role of the bgu operon in the utilization of arbutin or analogous sugars that S. pneumoniae encounters in its natural environment.

Deletion of the genes encoding the PTS components (bguDBC) of the bgu operon has no effect on growth compared to the wild-type in the presence of cellobiose as the sole carbon source, whereas deletion of celR (impairing expression of the cel locus) does. Recent studies in S. pneumoniae R6 have shown that deletion of bguD did lead to an increased lag-phase on cellobiose and a slight effect on growth on amygdaulin [17,18]. However, deletion of celD led to strong inhibition of growth of S. pneumoniae R6 on amygdaalin, cellobiose, esculin and gentiobiose [17]. Thus, the cel locus is primarily responsible for β-glucoside utilization in S. pneumoniae and the exact role of the bgu operon in the uptake and metabolism of β-glucosides remains to be determined.

Interestingly, the BguR operator site could only be found in the promoter region of the bguA gene. This suggests that the bgu operon is the only direct target of BguR. However, deletion of bguR also led to upregulation of maltose- and fructose utilization gene clusters. The mal gene cluster is regulated by malR, a maltose-dependent transcriptional repressor [30,46]. Therefore, the upregulation of the mal gene cluster might be an indirect effect of the high upregulation of the bgu operon. However, the regulatory mechanism of the putative fructose utilization gene cluster in S. pneumoniae has not yet been explored. It could well be that the LacR type regulator encoded in this gene cluster directly regulates it.

Supporting Information

Table S1 Overview of all significantly up- or downregulated in the presence of cellobiose, compared to growth in the presence of glucose, at time point T1 and T2.

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Author Contributions

Conceived and designed the experiments: SS OPK TGK. Performed the experiments: SS. Analyzed the data: SS. Contributed reagents/materials/analysis tools: SS OPK TGK. Wrote the paper: SS OPK TGK.

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