Regulation of neuraminidase expression in *Streptococcus pneumoniae*

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**Abstract**

**Background:** Sialic acid (N-acetylneuraminic acid; NeuNAc) is one of the most important carbohydrates for *Streptococcus pneumoniae* due of its role as a carbon and energy source, receptor for adhesion and invasion and molecular signal for promotion of biofilm formation, nasopharyngeal carriage and invasion of the lung.

**Results:** In this work, NeuNAc and its metabolic derivative N-acetyl mannosamine (ManNAc) were used to analyze regulatory mechanisms of the neuraminidase locus expression. Genomic and metabolic comparison to *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus gordonii* and *Streptococcus sanguinis* elucidates the metabolic association of the two amino sugars to different parts of the locus coding for the two main pneumococcal neuraminidases and confirms the substrate specificity of the respective ABC transporters. Quantitative gene expression analysis shows repression of the locus by glucose and induction of all predicted transcriptional units by ManNAc and NeuNAc, each inducing with higher efficiency the operon encoding for the transporter with higher specificity for the respective amino sugar. Cytofluorimetric analysis demonstrated enhanced surface exposure of NanA on pneumococci grown in NeuNAc and ManNAc and an activity assay allowed to quantify approximately twelve times as much neuraminidase activity on induced cells as opposed to glucose grown cells.

**Conclusions:** The present data increase the understanding of metabolic regulation of the *nanAB* locus and indicate that experiments aimed at the elucidation of the relevance of neuraminidases in pneumococcal virulence should possibly not be carried out on bacteria grown in glucose containing media.

**Keywords:** Sialic acid, Metabolic regulation, Carbon catabolite repression

**Background**

*Streptococcus pneumoniae* is a common inhabitant of the upper respiratory tract and it is also a major human pathogen. The self-limited carriage episodes represent the most common interaction between pneumococci and the host. However, in some cases, such asymptomatic interaction can progress to invasive disease [1]. Of the many factors influencing the interaction of the bacterium with the host, numerous extracellular glycosylhydrolases and carbohydrate transporters have been found to play significant roles [2]. The sialidases or neuraminidases, which are able to cleave terminal sialic acid (neuraminic acid, NeuNAc) residues present in O-linked and N-linked glycans, have since long received special attention as virulence determinants [3,4]. Direct interaction of the microbial sialidases with host glycoproteins resulting in exposure of additional attachment sites on host cells was the mechanisms most frequently found to be involved in virulence [5-7]. Recently such interaction was found to be directly involved in invasion [8,9]. Despite the impact of sialidases in pneumococcal pathogenesis, metabolic implications have received less attention, including the utilisation of sialic acid as a carbon source on the glucose-free mucosal surfaces [10-16]. Sialic acid has recently been described by us and others to act as a molecular signal for pneumococci, resulting in increased carriage and translocation of bacteria to the lung [10,14,17].

Given the prominent role of sialidases in host-pathogen interaction, it is not surprising that pneumococci harbour three sialidases, two of which, NanA and NanB, are common to all pneumococci and the third,
NanC, is present in only 51% of strains [18]. Structural and functional analysis of the three enzymes indicated possible different roles. NanA is a first-line virulence factor for sialic acid removal, the trans-sialidase NanB is involved in the metabolic use of sialic acid, and NanC has a regulatory role, being able to produce and remove an intermediate metabolic compound which also acts as sialidase inhibitor [19,20]. The conserved nanAB locus that comprises the genes between SPG1583 and SPG1601 in strain G54 (SP1674-94 in TIGR4) was identified as the cluster responsible for uptake and metabolism of sialic acid [16,21-23]. In addition to the extracellular sialidases NanA and NanB, the regulon encodes two ABC transporters, one of which responsible for sialic acid and N-acetyl mannosamine uptake SPG1589-91 (satABC) and the other (SP1596-8) for uptake of N-acetyl mannosamine alone [14,23]. In addition to the ABC transporters the locus encodes a PTS uptake system for glucosamine, and the remaining genes encode for enzymes involved in sialic acid metabolism [23]. 

In vitro this operon was found to be the main cluster showing differential expression in pneumococcal opacity variants and was predicted to be composed of four predicted transcriptional units [21]. During infection, the nanAB operon was found to be upregulated in pneumonia and meningitis compared to growth in blood [24,25].

Much less information is available on the nanC operon, except for the analysis of the enzymatic function of the sialidase NanC [20] and its recent implication as an alternative system for the uptake of sialic acid [23].

The present work aims at performing a functional analysis of the operon in order to gain further insight into the metabolic regulation of this locus.

**Results**

**The NanAB locus conservation in oral streptococci**

As a first approach to elucidate the metabolic relevance and regulation of the different predicted transcriptional units of the nanAB regulon, we performed a genomic comparison amongst related streptococcal species, including pneumococcal strain G54, S. mitis B6, S. oralis Uo5, S. sanguis SK36 and S. gordonii V288 (Figure 1A and Table 1). With respect to S. pneumoniae G54, S. mitis B6 and S. oralis Uo5, these showed an identical organization for part of the locus including the neuraminidase A (nana), the orthologs of the satABC transporter SPG1589-91 and the genomic regions encoding the transcriptional regulator and orthologues of the enzymes involved in the first steps of sialic acid metabolism, i.e. N-acetylneuraminate lyase and N-acetylmannosamine kinase (Figure 1). In contrast to pneumococci these two species, S. mitis and S. oralis, did not possess the sialidase NanB, the second ABC transporter SPG1596-8, and the PTS system. In contrast to S. mitis and S. oralis, S. gordonii V288 and S. sanguinis SK36 did not possess any neuraminidases. Interestingly both S. gordonii and S. sanguinis still possess orthologs of the N-acetyleneuraminate lyase, N-acetylmannosamine kinase and N-acetylmannosamine-6-phosphate 2-epimerase predicted to be necessary for metabolism of sialic acid (Figure 1A,B; Table 1).

In addition, S. gordonii and S. sanguis possessed the transcriptional regulator and the orthologs of the pneumococcal SPG1596-8 ABC transporter. In contrast to S. pneumoniae, S. gordonii and S. sanguis possess neither the PTS system nor the SPG1589-91 satABC transporter.

To check the amino sugar metabolism of these three different species of streptococci growth curves and fermentation assay on NeuNAC and ManNAC were performed. The growth curves show that S. gordonii grows only in presence of ManNAC, while S. mitis and S. pneumoniae are capable of growth on both amino sugars (Figure 2A,C). Similarly in the fermentation assay only S. gordonii acidified efficiently the medium in presence of ManNAC, while both S. pneumoniae and S. mitis metabolised efficiently only NeuNAC, with some acidification of the medium with ManNAC by the pneumococcus (Figure 2D).

**Neuraminidase locus induction in S. pneumoniae**

The putative regulator of the nanAB locus SPG1583 contains a classical N-terminal helix-turn-helix motif and a SIS domain, found in many phosphosugar binding proteins including transcriptional regulators binding to the phosphorylated end-products of the pathways [26].

Given the probable catabolic pathway of sialic acid (Figure 1B), ManNAC-6-phosphate appears to be the most probably compound having a regulatory role on the expression of pneumococcal neuraminidase operon and thus possibly in sialic acid metabolism [23]. Therefore we analysed the growth curves and the expression levels of some key genes associated with the transporter systems in the neuraminidase locus. First we compared the growth in the presence of ManNAC as a carbon source of a un-encapsulated G54 derivative FP65 and two isogenic mutants devoid of the whole nanAB locus and of the transcriptional regulator SPG1583 respectively (Figure 3A). The growth curves showed absence of growth in the presence of ManNAC for both mutants, indicating that the nanAB locus is essential for efficient growth of ManNAC and that the phosphosugar binding regulator SPG1583 gene appears to acts as a transcriptional activator. Then we focused our attention on growth of the wild type strain in the presence or absence of ManNAC, preferred by us for the indication assays over NeuNAC, as this amino sugar does not acidify the medium. In these experiments bacteria initially grew on residual yeast-extract derived dextran of non-supplemented CAT medium (40 min) and continued to
grow thereafter with a lower generation time of 140 min on ManNAc only (Figure 3B). For gene expression profiling bacteria were sampled in early exponential growth (OD$_{590}$ = 0.02), when growth was still due to the residual yeast extract-derived sugar (Figure 3B, black arrows). For bacteria grown on yeast extract derived sugar in presence of ManNAc, gene expression data showed a significant induction of the \textit{satABC} (SPG1589-91 and SPG1592) PTS transporters, and a non-significant induction of \textit{nanA} (Figure 3C). We performed a second experiment that compared the influence of ManNAc at OD$_{590}$ = 0.02 and 0.05 on gene expression (Figure 3B, open arrows). This assay compared bacteria grown on yeast-derived sugar and ManNAc, where the first time point represents growth on yeast-extract derived sugar (in presence of ManNAc) and the second growth on ManNAc after termination of the other carbohydrates. Growth on ManNAc caused a significant increase of transcriptional levels of all genes analysed (Figure 3D). The values of mean fold changes were 17.61 (p < 0.01) for \textit{nanA}, 52.18 (p < 0.01) for SPG1598, 6.33 (p < 0.05) for SPG1592 and 6.65 (p < 0.05) for \textit{satC} SPG1591.

To evaluate the role of glucose and of the two amino sugars ManNAc and NeuNAc in the regulation of the \textit{nanAB} regulon, we quantify gene expression during growth in the presence of these sugars. Bacteria were grown in the presence of ManNAc (Figure 4A, open triangles) or NeuNAc (Figure 4B, open triangles) and their gene expression was compared to that of bacteria grown with 1 g/L glucose alone (Figure 4A,B, closed circles). All genes of the \textit{nanAB} regulon showed a significant increase in transcription in presence of any of the amino sugars. The values of mean fold changes were: \textit{nanA}, 2.69 (p ≤ 0.05) in ManNAc and 5.14 (p ≤ 0.05) in NeuNAc; SPG1598, 3.35 (p ≤ 0.05) in ManNAc and 1.99 in NeuNAc; SPG1592, 3.21 (p ≤ 0.05) in ManNAc and 3.74 (p ≤ 0.05) in NeuNAc; SPG1591, 3.45 (p ≤ 0.05) in ManNAc and 5.13 (p ≤ 0.01) in NeuNAc. Interestingly the
transporter SPG1596-8 linked to the growth and fermentation of ManNAc was more induced by this sugar, while NeuNAc had a significantly greater effect on the satABC SPG1589-91 transporter, again in accordance with phenotypic data.

Repression of the nanAB locus in the presence of glucose

According to the presence of three cre sites within the pneumococcal neuraminidase locus, we observed a biphasic growth curve when bacteria grew on glucose plus ManNAc or NeuNAc (Figure 4A,B, open squares). To demonstrate that this phenotype was due to carbon catabolite repression, we investigated the transcriptional behaviour of the neuraminidase locus in the presence or absence of glucose in the medium. Growth conditions used were as follows: ManNAc with and without glucose (Figure 4A, open triangles and open squares), NeuNAc with and without glucose (Figure 4B, open squares and open diamonds) and glucose as the sole carbon source as a reference condition (Figure 4A and 4B, closed circles). Growth curve data show that addition of glucose to both ManNAc and NeuNAc resulted in an initial growth on glucose as a preferred carbon source followed by a second slower growth phase, in which the amino sugars were metabolised. To assess glucose repression during growth on glucose gene expression analysis was carried out by sampling the bacteria at an OD590 of 0.05 (Figure 4A,B, arrows). As shown in Figure 4C, the overexpression of all genes of the nanAB locus occurred during growth on ManNAc or NeuNAc as the sole carbon sources (Figure 4C, open and grey bars), while it was completely repressed in the presence of glucose (Figure 4C, striped bars).

Regulation of neuraminidase A production and activity by ManNAc

To assess the production of NanA on the bacterial surface after induction of the nanAB locus by ManNAc or NeuNAc, we performed a cytofluorimetry assay. In these experiments bacteria were harvested at the late exponential phase. In this assay the anti-NanA serum recognises also to a certain extent glucose grown bacteria (Figure 5A). However in culture media with either ManNAc or NeuNAc as the sole carbon sources, the number of NanA expressing bacterial cells significantly increased reaching 73.7% (± 3.4) and 79.6% (± 4.9), respectively. Differences in NanA production between bacterial cells grown with either of the two amino sugars and control cells cultured in glucose or glucose plus ManNAc were statistically significant (Figure 5A). For more detailed analysis a fluorescence assay for the detection of cell surface-associated neuraminidase activity was carried

### Table 1 List of gene annotation in the nanAB locus

| Annotation | Figure 1A* | S. pneumoniae TIGR4 | S. pneumoniae G54 | S. mitis B6 | S. oralis Uo5 | S. gordonii V288 | S. sanguinis SK36 |
|------------|------------|----------------------|-------------------|-------------|---------------|------------------|-----------------|
| Regulator  | 1          | SP1674               | SPG1583           | smi0612     | SOR0560       | SGO0127          | SSA0081         |
| Hypothetical protein | 2          | -                    | -                 | smi0610     | SOR0559       | SGO0126          | SSA0080         |
| N-acetylmannosamine kinase | 3          | SP1675               | SPG1584           | smi0609     | SOR0558       | SGO0125          | SSA0079         |
| N-acetylneuraminate lyase | 4          | SP1676               | SPG1585           | smi0608     | SOR0557       | SGO0124          | SSA0078         |
| hypothetical protein | 5          | SP1677               | SPG1586           | smi0607     | SOR0556       | -                | -               |
| hypothetical protein | 6          | SP1679               | -                 | -           | -             | -                | -               |
| hypothetical protein | 7          | SP1680               | SPG1588           | smi0606     | SOR0555       | -                | -               |
| satA ABC transporter permease | 8          | SP1681               | SPG1589           | smi0605     | SOR0553       | -                | -               |
| satB ABC transporter permease | 9          | SP1682               | SPG1590           | smi0604     | SOR0552       | -                | -               |
| satC ABC transporter substrate-binding protein | 10         | SP1683               | SPG1591           | smi0603     | SOR0550       | -                | -               |
| PTS system, IIIBC components | 11         | SP1684               | SPG1592           | -           | -             | -                | -               |
| NanE, ManAc-6P 2-epimerase | 12         | SP1685               | SPG1593           | smi0602     | SOR0549       | SGO0118          | SSA0071         |
| oxidoreductase | 13         | SP1686               | SPG1594           | -           | -             | SGO0123          | SSA0077         |
| NanB neuraminidase | 14         | SP1687               | SPG1595           | -           | -             | -                | -               |
| ABC transporter permease | 15         | SP1688               | SPG1596           | -           | -             | SGO0122          | SSA0076         |
| ABC transporter permease | 16         | SP1689               | SPG1597           | -           | -             | SGO0121          | SSA0075         |
| ABC transporter substrate-binding protein | 17         | SP1690               | SPG1598           | -           | -             | SGO0120          | SSA0074         |
| hypothetical protein | 18         | SP1691               | SPG1599           | -           | -             | SGO0119          | SSA0073         |
| NanA neuraminidase | 19         | SP1693               | SPG1600           | smi0601     | SOR0548       | -                | -               |
| Acetyl xylan esterase | 20         | SP1694               | SPG1601           | smi0600     | SOR0547       | -                | SSA0070         |

* numbers as in Figure 1A.
out to investigate the dependence of neuraminidase production upon the nature of the carbon source provided during bacterial growth (glucose vs N-acetylmannosamine). The amount of neuraminidase activity in cell samples containing $10^7$ CFU/ml was clearly higher (approx. 12-fold) in the presence of N-acetylmannosamine rather than glucose (Figure 5B), indicating that N-acetylmannosamine is an inducer of neuraminidase production while in glucose grown cells neuraminidase activity is clearly repressed. $10^7$ CFU of \textit{S. pneumoniae} FP65 grown in the presence of N-acetylmannosamine yielded a neuraminidase activity equivalent to that of 7.5 μg of purified NanA, indicating that this strain produces a significant amount of neuraminidase(s) in the presence of amino sugars. These numbers are such to propose approximately 100–500 enzymes per cell when bacteria are grown in amino sugar and only few enzymes per cell when bacteria are grown in glucose.

**Discussion**

Pneumococcal neuraminidases are the most studied surface located glycosyl-hydrolases due to their role in pathogenicity as factors involved in adhesion and invasion of \textit{S. pneumoniae} to host cells [5,6,8,9,27,28]. In addition, their role in the release of free sialic acid from oligosaccharides has been proposed as an important source of carbon and energy [13,14,29,30]. More recently, the cleavage of sialic acid from O-glycans has been related to pathogenesis, by proposing sialic acid as a molecular signal to promote in vitro biofilm production and in vivo nasopharyngeal carriage and lung invasion by \textit{S. pneumoniae} [10,17]. In this context, the regulatory mechanisms of the neuraminidase locus expression are of importance. So far nearly all data on virulence and expression of the two loci containing neuraminidases has been carried out on the \textit{nanAB} locus only, since the D39 reference strain does not carry the \textit{nanC} locus [18]. The main finding on expression of the \textit{nanAB} locus reported its organisation in four predicted transcriptional units, of these the one harbouring NanA and the one encoding for the enzymes of the sialic acid metabolism were differentially expressed in transparent and opaque pneumococcal colony variants [21]. Additionally the increased expression of this locus during infection [10,24,25], further underlines the importance of neuraminidases in the interaction of pneumococci with the host. It should be noted that most

**Figure 2** Metabolic utilisation of ManNAc and NeuNAc by \textit{S. gordonii}, \textit{S. mitis} and \textit{S. pneumoniae}. \textit{S. gordonii} V288 (A), \textit{S. pneumoniae} G54 (B), and \textit{S. mitis} NCTC12661 (C) were grown in CAT medium (200 U catalase) supplemented with 2 g/L glucose (black line) 2 g/L ManNAc (red line) and 1.5 g/L NeuNAc (blue line). CAT medium alone as a source of carbon is in grey line. All strains were grown for 38 hours at 37°C in 200 μl of medium in a 96 well microplate with reading intervals of 10 min. For the fermentation assay (panel D) bacteria were incubated for 24 and 48 h with serial dilutions of either ManNAc (left columns) or NeuNAc (right columns) as sole carbon sources in microtiter plates containing phenol red as a pH indicator. Sugar fermentation is evidenced by a yellow colour change due to acidification of the culture medium. Carbohydrate concentrations (% w/v) are shown on the right.
of the above work on pneumococcal virulence is done utilising strain D39, which is unable to ferment sialic acid due to a frame shift in the neuraminate lyase of the \textit{nanAB} locus \cite{23,31}, a fact which apparently does not influence regulation of the locus and virulence of the bacterium.

We have recently shown that the two ABC transporters of the \textit{nanAB} locus, and also the sodium symporter of the \textit{nanC} locus to a lesser extent, are not only involved in sialic acid uptake, but also in the transport of ManNAc, which represents the first metabolic intermediate in pneumococcal NeuNAc catabolism \cite{23}. In this work we focus our attention on the contribution of the \textit{nanAB} locus, since deletion mutants for the \textit{nanC} locus had been shown not to influence growth on ManNAc and NeuNAc during the first 18–24 hours of incubation, implying a limited or absent regulatory crosstalk between the two regulons \cite{14,23}. The two ABC transporters were shown to be able to support growth on amino sugars, with SPG1596-8 and SPG1589-91 being the main transporters for ManNAc and NeuNAc, respectively \cite{23}. In this work we have combined genomic information, gene expression and growth phenotypes to further clarify these data. When performing \textit{in silico} analysis of the \textit{nanAB} locus we observed the presence of part of the locus in related oral streptococci. Here we utilised this genomic information to strengthen the correlation between orthologous transporters and metabolic functions. \textit{S. sanguinis} and \textit{S. gordonii}, harbouring an operon including the orthologue of the SPG1596-8, were found to be able to efficiently metabolise ManNAc, but not NeuNAc. To the contrary \textit{S. mitis} and \textit{S. oralis}, which are much more closely related to pneumococci, harboured a locus, in addition to all the metabolic genes, also encoding for a neuraminidase and the orthologue of the \textit{satABC} SPG1589-91 transporter \cite{14}. The finding that \textit{S. mitis} can efficiently metabolise NeuNAc and ManNAc, confirm that the substrate specificity identified

**Figure 3 Growth and induction of gene expression by ManNAc.** (A) Growth of \textit{S. pneumoniae} strains on CAT medium supplemented with 10 g/L of ManNAc: FP65 (open squares), \textit{nanAB}-deficient mutant (open triangles), and SPG1583-regulator deletion mutant (closed circles). (B) Growth of FP65 on CAT medium without added sugar (closed squares) and supplemented with ManNAc 10 g/L (open squares). The white and black arrows indicate samples taken for quantitative Real Time-PCR. Gene expression analysis of the genes coding for NanA the ABC transporter SPG1598, the PTS transporter SPG1592, and the ABC transporter SPG1591 is shown in panel C and D. Panel C refers to fold changes in transcriptional levels at OD 0.02 in medium with or without ManNAc (for sampling see closed arrows in panel 3B). Panel D refers to analysis of sequential samples (OD$_{590}$ = 0.02 and OD$_{590}$ = 0.05) of bacteria grown in ManNAc (for sampling see open arrows in panel 3B). The fold changes are reported as mean from independent triplicate or quadruplicate experiments. Two-tailed Student \textit{t} test was used for analyse statistical significance (*, p < 0.05; **, p < 0.01). Generation time on unsuplemented CAT medium is 40 min and on ManNAc 140 min.
for the pneumococcal transporters is generally well conserved in orthologues of related species [14]. Interestingly, all oral streptococci share the core part of metabolic enzymes of the operon, suggesting comparable capability to metabolise both NeuNAc and ManNAc. These observations match earlier data that described detectable levels of metabolism of NeuNAc in most oral streptococci, while sialidase activity could only be found in few species [32]. Amongst the oral streptococci, pneumococci carry a composite locus, probably assembled from the gene pool of related species. The association of the SPG1594 oxidoreductase with ManNAc metabolism and of two small hypothetical proteins (SPG1586 and SPG1588) with NeuNAc metabolism remains unexplained, as all necessary enzymes for sialic acid metabolism appear to be already present. The PTS transporter, found to transport glucosamine, appears to be unique in pneumococci [23]. The fact that glucosamine is the last metabolic intermediate in sialic acid catabolism may indicate a convenience for the bacterium in co-utilisation of GlcN and ManNAc, even if it is not clear where pneumococci should feed on GlcN, a rare sugar in the human nasopharynx, but of which on the contrary the pneumococcal cell wall is exceptionally rich [33].

When pneumococci grow on ManNAc and NeuNAc as the sole carbon sources, the generation time is much longer than on glucose or on the yeast-extract derived carbohydrates of the CAT medium, which is in accordance with previous data [23]. Growth on ManNAc (Figure 3B, Figure 4A) shows a profile with a change in generation time. In the case of growth on glucose repression of the whole locus indicates sequential utilisation of sugars. This is less clear for the growth on yeast derived sugars, where only part of the locus is induced with the exception of the predicted central transcriptional unit encoding the principal ManNAc ABC transporter SPG1596-8. The data here presented thus do not rule out, that during growth on yeast derived sugars also ManNAc may be co-metabolised. The differential impact of regulation on the three operons is reminiscent of data on expression of this locus in transparent colony variants, where also the nanB and ManNAc-uptake operon is not involved in differential expression, while the other two transcripts
are upregulated [21]. The fact that both ManNAc and NeuNAc are able to efficiently induce the operon is in accordance with our finding that the SPG1583 regulator acts as a positive regulator, as documented by the absence of metabolism in its mutant and also by its annotation as a phosphor-sugar binding regulator. Since NeuNAc is imported by an ABC transporter, which does not phosphorylate during uptake, and is first hydrolysed to ManNAc before becoming phosphorylated (Figure 1B), both amino sugars may equally originate the inducer of the positive regulator; probably ManNAc-phosphate.

The sequential utilization of carbon sources is generally regulated by carbon catabolite repression, and in bacteria it has been linked not only to metabolic use, but also to more general mechanisms involved in host-pathogen interactions [34,35]. As in other Gram-positive bacteria, also in S. pneumoniae carbon catabolite repression involves the carbon catabolite control protein A (CcpA) which regulates operons by binding to a specific operator sequence, named as catabolite-repressible element (cre site) [36-39]. Multiple cre sites were recently predicted upstream SPG1560, SPG1597 and SPG1593 in the nanAB locus [37,38], and array analyses proved the role of CcpA in its regulation and interestingly relief of ccpA repression shows much more pronounced effects on the “NeuNAc operon” (SPG1599-84) than on the “ManNAc operon” (SPG1599-4). The cre sites and CcpA-mediated regulation is in accordance with the transcriptional units described earlier [21]. Our data here confirm that glucose completely represses the expression of all three predicted transcriptional units of the nanAB locus. The above gene expression data are also consistent with the neuraminidase activity assay on whole cells, which indicates twelve times more enzymatic activity in induced cells with respect to glucose grown cells. The repression of both neuraminidases and the intracellular enzymes for sialic acid metabolism had already been reported for a large number of viridians streptococci, which thus share with S. pneumoniae a strong effect of carbon catabolite repression on the loci responsible of NeuNAc metabolism [32].

Conclusions
In summary, the data obtained in our study confirmed and demonstrated that, (i) pneumococci carry a composite locus, in part shared by related species, which is predicted to metabolise both ManNAc and NeuNAc, (ii) pneumococci could use both ManNAc and NeuNAc as the sole carbon sources for growth, (iii) uptake of ManNAc and NeuNAc involved preferentially the SPG1596-8 and the satABC SPG1589-91ABC transporters, respectively, (iv) ManNAc and NeuNAc could

![Figure 5](http://www.biomedcentral.com/1471-2180/12/200)

**Figure 5 Neuraminidase protein production and activity on whole cells.** A cytofluorimetric assay with an anti-NanA serum was performed on pneumococci grown on different carbohydrates (panel A). The presence of NanA at the bacterial surface was tested in samples cultivated in glucose (open bar), glucose + ManNAc, ManNAc alone (grey bar), and NeuNAc alone (black bar) (all carbohydrates were at 1 g/L). Data are represented as mean values ± SD of percent bacterial population positive for NanA production and derived from quadruplicates experiments performed independently. Asterisks (*, p < 0.05; **, p < 0.001) indicated statistical significance. Panel B shows the hydrolysis of Z’-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (4MU-Neu5Ac) in the presence of 40 µl S. pneumoniae FP65 cell samples grown in CAT medium with either glucose (white circles) or N-acetylmannosamine (black circles). The neuraminidase activity was computed as the variation of fluorescence vs time using a linear regression of the data (dashed lines). Inlet. Hydrolysis of 4MU-Neu5Ac by purified NanA neuraminidase, showing the proportionality between enzyme concentration and rate of fluorescence variation. Enzyme concentrations were 10 nM (black circles), 20 nM (triangles), 30 nM (diamonds) and 40 nM (squares). The empty circles show the variation of fluorescence vs time for the substrate alone.
induce the *nanAB* locus, which is subjected to carbon catabolite repression by glucose and (v) a quantitative neuraminidase activity assay allowed to tentatively quantify neuraminidases on the surface of pneumococci grown in amino sugars to numbers around 100–500 enzymes per cell. Interestingly, some growth conditions were found to mimic the transcriptional profile observed for pneumococcal transparent colony variants, suggesting a metabolic influences on pneumococcal phase variation [21]. Still, the differential induction of the predicted transcriptional units by the two amino sugars, indicates that probably carbon catabolite repression and activation by the regulator act at different strength on the three transcriptional units. Finally as already shown in oral streptococci [32], the amount of NanA significantly increases and neuraminidase activity during growth on ManNAc or NeuNAc, indicating that experimental conditions based on mid log glucose-grown bacterial cells may be biased in estimating the actual contribution of neuraminidases to host-pathogen interaction.

**Methods**

**Bacterial strains and culture media**

Bacterial strains used in this work were *S. pneumoniae* strain G54 (serotype 19F) and its un-encapsulated derivative FP65 [40], since the pneumococcal reference strain D39 has a frame shifted neuraminate lyase gene and TIGR4 did not grow efficiently in CAT medium [23]. Most experiments are performed with the un-encapsulated FP69 as strains without are non virulent and no influence on sugar metabolism has been observed (data not shown). Oral streptococci where *S. mitis* NCTC12261 (kindly provided by Morgens Kilian) and *S. gordonii* V288 Challis [41]. Bacteria were plated on Tryptic soy agar plates (TSB; Liofilchem Roseto degli Abruzzi, Italy) containing 3% v/v glucose non-supplemented CAT medium either without added sugar or with addition of either glucose, ManNAc, NeuNAc, glucose + ManNAc, or glucose + NeuNAc, all at 1 g/L. Bacterial growth curves were performed in 96-well plates in a thermostated spectrophotometer at 37°C. Plates were shaken gently for 10 seconds prior to each reading, and the optical density was read automatically in 10 min intervals.

**Carbohydrate fermentation**

The method for evaluation sugar uptake and fermentation has been recently described [23]. Briefly, bacteria grown on agar plates were resuspended to an OD590 = 0.6 in CAT medium and diluted 1:1 with CAT medium supplemented with K2HPO4, the appropriate sugar and catalase as reported above. After o.n. incubation, pH changes were visualised by addition of phenol red (0.1 mg/ml) (P4633 Sigma-Aldrich).

**Growth curve and sample collection**

In order to characterize the gene expression pattern in a specific point of the growth curve, we sampled bacteria during growth. Strains were grown on TSA plates at 37°C in a CO2 enriched atmosphere for 18 hours. Bacteria were then collected with a swab and resuspended at the OD590 of 0.2 in non-supplemented CAT medium. Bacterial samples were diluted 1:100 in CAT medium either without added sugar or with addition of either glucose, ManNAc, NeuNAc, glucose + ManNAc, or glucose + NeuNAc, all at 1 g/L. Bacterial growth curves were performed in 96-well plates in a thermostated spectrophotometer at 37°C. Plates were shaken gently for 10 seconds prior to each reading, and the optical density was read automatically in 10 min intervals
Neuraminidase activity
The neuraminidase activity was measured using the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4MU-Neu5Ac) (M8639, Sigma-Aldrich, St. Louis, Miss.). The time dependence of the variation of fluorescence (\(\Delta A_{600} = 0.4\) in 10 mM MES buffer pH 6.0, 1 final A\(600\) = 0.4 in 10 mM MES pH 6.0. The method was initially optimized and calibrated using purified NanA neuraminidase of \(S.\ pneumoniae\) D39 produced in \(E.\ coli\) (0.88 mg/ml) (data not shown). The activity was computed as the variation of fluorescence vs time using a linear regression of the data. In our conditions, 1 μg of purified NanA yielded a activity of 10,690 ΔF/min.

Abbreviations
NeuNAc: Sialic acid (N-acetylneuraminic acid); ManNAc: N-acetylmannosamine; ABC transporter: ATP binding cassette transporter; PTS transporter: Phosphotransfer system transporter; CAT medium: Casitone yeast extract medium.

Competing interests
The author declare that they have no competing interests.

Authors' contributions
LG, JKH, AG, AB, LC, and CT generated data in the laboratory and Biomedical Science, University of Adelaide, South Australia 5005, Australia.

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