Fructooligosaccharide Utilization by *Streptococcus pneumoniae*

Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation with honors research distinction in Microbiology in the undergraduate colleges of The Ohio State University

By

Caroline Linke

The Ohio State University

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Project Advisor: Samantha J. King, Department of Pediatrics

Abstract

Streptococcus pneumoniae (pneumococcus), a leading cause of morbidity and mortality worldwide, commonly colonizes the oronasopharynx asymptomatically but can on occasion progress beyond this site and cause disease. Pneumococcal colonization is a necessary precursor to disease and is dependent upon the ability of pneumococci to acquire and utilize carbohydrates. There are two main types of carbohydrate transporters encoded in the pneumococcal genome: the Phosphotranferase Systems (PTS) and the ATP-Binding Cassette (ABC) transporters. There are two proposed sucrose transporters encoded within the pneumococcus genome, a PTS, ScrT, and an ABC transporter, SusT1T2X. As ABC transporters are less energetically favorable than the PTS and are predicted to be subjected to catabolite repression, we hypothesize the main substrate of the SusT1T2X is not sucrose. The presence of a gene encoding sucrose hydrolase in the same genomic locus as *susT1T2X* led us to believe the main substrate of this transporter is sucrose-containing. Inulin is a fructooligosaccharide (FOS) consisting of a long chain of fructose molecules with a terminal sucrose. Inulin is fermented by approximately 60% of pneumococcal strains yet the mechanism of utilization remained unknown. We have shown both inulin and shorter chains of FOS can support growth of the pneumococcal strain TIGR4 as a sole carbon source. An unmarked, nonpolar deletion of *susT1T2X* was created. This strain, TIGR4 Δ*susT1T2X*, was unable to utilize FOS longer than a glucose linked to three fructose. A genetically reconstituted strain was able to restore growth on these FOS indicating the *sus* locus is required for import of FOS. Furthermore, it has previously been reported that pneumococcal strains encode one of two distinct ABC transporters at the *sus* locus. Data suggest that while both transporters encoded at the *sus* locus allow for the import of short chain FOS, only one of the transporters allow for the import of long chain FOS, or inulin.

Acknowledgments

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CONTENTS

Introduction

Streptococcus pneumoniae is a Gram positive, human pathogen that commonly colonizes the nasopharynx and is a major cause of disease worldwide. These diseases include pneumonia, otitis media, sinusitis, bacteremia, meningitis, and endocarditis. Current vaccines target the capsular polysaccharide of *S. pneumoniae* and because of this, they provide stereotype specific protection. *S. pneumoniae* is a very diverse species with over 90 different stereotypes making it difficult to provide protection against all strains (20). In addition, treatment of disease is becoming increasingly challenging due to rising numbers of antibiotic resistant *S. pneumoniae* isolates (31). Therefore, there is an interest in developing more effective vaccines and therapeutics.

Pneumococcal colonization is often asymptomatic, but colonization is a necessary precursor to pneumococcal disease. *S. pneumoniae* requires carbohydrates for growth and therefore colonization, yet relatively little is known about how the bacteria acquire and utilize carbohydrates. Free carbohydrates are relatively scarce in the nasopharynx, but the pneumococcus has developed ways of utilizing complex carbohydrates. It is known that pneumococci can cleave complex glycans that are found on the surface of human cells and as these carbohydrates have been shown to support pneumococcal growth in vitro, it is likely they do so in vivo as well (17, 23). It is also possible carbohydrates in the capsule of other bacteria are used as a carbon source in vivo as it has been shown that carbohydrates isolated from the capsule of bacteria can support pneumococcal growth in vitro (24).

Two main types of carbohydrate transporters have been identified in the *S. pneumoniae* genome: the Phosphotranferase Systems (PTS) and ATP-Binding-Cassette (ABC) transporters. The strain TIGR4 is predicted to encode 17 complete PTS and six ABC CUT1 transporters (35). ABC CUT1 transporters are made up of a membrane bound substrate binding protein, membrane spanning permeases that form a channel through which the substrate enters the cell and two intracellular ATPases that bind to the permeases and hydrolyze ATP to energize transport. PTS transporters require less energy than the ABC transporters so the substrates of these transporters are predicted to be preferred carbohydrates. Because of this, the ABC carbohydrate transporters in *S. pneumoniae* are predicted to be subjected to catabolite repression under favorable growth conditions.

There are two predicted sucrose transporters encoded by pneumococci, a PTS, encoded by *scrT* and an ABC encoded by *susT1T2X* (Fig. 1) (15).

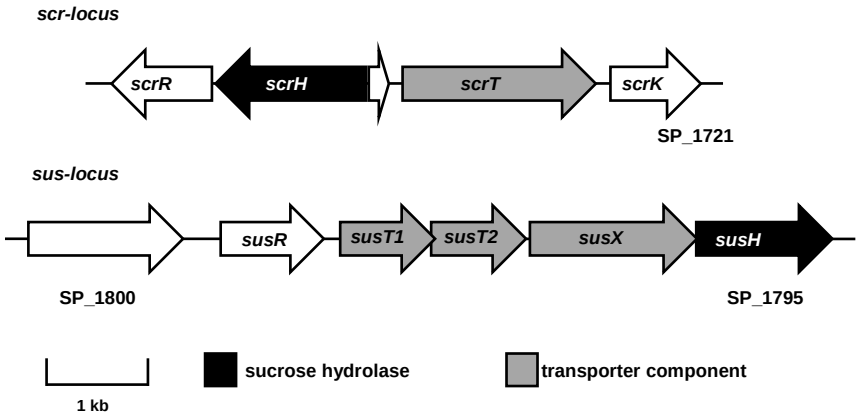

Figure 1. Schematic of the two putative sucrose utilization operons encoded by the *S. pneumoniae* strainTIGR4. Open reading frames predicted within the TIGR4 sequence are represented by block arrows.

susT1 and susT2 are predicted to encode the two permeases and *susX* is predicted to encode the substrate binding protein. Directly downstream of these genes is a gene encoding a predicted

sucrose hydrolase, SusH. Also in the same genomic locus is a predicted LacI family regulator, SusR. Interestingly, lacking in the genomic region is a gene(s) encoding an ATPase(s), an essential components of the transporter. All six ABC CUT1 transporters encoded by TIGR4 lack a gene(s) encoding an ATPase(s) in their genomic loci and the gene *msmK* is predicted to encode for the ATPase that energizes all six transporters (21). Because ABC transporters are believed to be repressed under favorable growth conditions, or when carbohydrates are being brought into the cell through the PTS, it seems unlikely sucrose is the primary substrate of both a PTS and an ABC transporter. If there were both a sucrose PTS and ABC transporter, the ABC would be predicted to be subjected to catabolite repression when its substrate is present.

ScrT and SusT1T2X were initially identified as sucrose transporters based on the presence of genes in their loci encoding glycosyl hydrolase 32 family proteins that contain a signature β-fructosidase motif (NDPNG) and can contribute to growth on sucrose (11, 12, 15). It was shown that in the absence of either hydrolase, the other could compensate (15). It was subsequently shown that growth of a strain lacking *scrT* was reduced but not eliminated on sucrose. Growth of a strain lacking the substrate binding protein *susX* was not reduced on sucrose but a strain lacking both *scrT* and *susX* showed further reduction over the single mutant (4). This indicates that ScrT is the main sucrose transporter and that while SusT1T2X may contribute to sucrose transport in vitro, the main substrate of this transporter is not sucrose. This is further supported by the finding that sucrose induces expression of the *scr* locus but not the *sus* locus (15). In addition, it has previously been reported that *sus* locus contributes to in vivo fitness in the lung and not the nasopharynx suggesting the two transporters import different carbohydrates (15). Studies in our lab, using the strain TIGR4, we have shown the same phenotype as previously

demonstrated for the single mutants, but we have not seen consistent further reduction of growth on sucrose of TIGR4 Δ*scrt* Δ*susT1T2X* over TIGR4 Δ*scrt* (TIGR4 Sm^r maximal OD_{600} nm ≥ 0.69, TIGR4 Δ*scrt* maximal $OD_{600nm} \leq 0.44$, TIGR4 Δ*scrT* Δ*susT1T2X*, maximal $OD_{600nm} \geq 0.52$) (Fig 2).

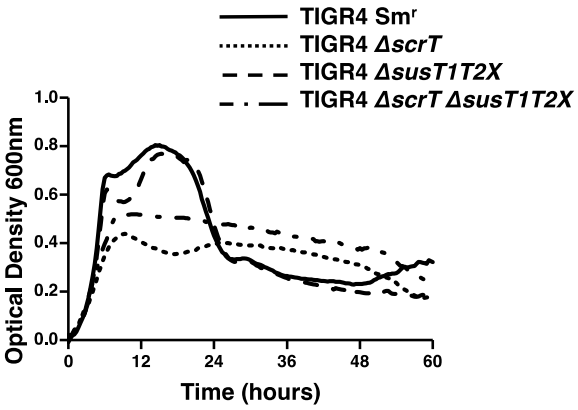

Figure 2. *scrT* contributes to growth on sucrose. TIGR4 Sm^r, TIGR4 Δ*susT1T2X*, TIGR4 Δ*scrT* and TIGR4 Δ*scrT* Δ*susT1T2X* were grown for 60 h on chemically defined medium (CDM) supplemented with 12 mM sucrose as the sole carbon source. Growth was measured by the optical density at 600 nm. A CDM no sugar control has been subtracted from each data set. This experiment was performed at least three independent times in triplicate and the OD_{600} values represent one experiment.

While we propose the main substrate of SusT1T2X is not sucrose, the presence of the gene encoding a sucrose hydrolase within the predicted operon leads us to believe the main substrate of this transporter has a similar structure to sucrose.

Inulin is a fructooligosaccharide (FOS) and naturally occurring fiber that acts a prebiotic. It is a chain of fructose molecules joined by β(2-1) glycosidic bonds with a terminal α(1-2) glucose molecule. The terminal α(1-2) linked glucose and fructose is a sucrose molecule. It has been reported that about 60% of pneumococcal strains can ferment inulin (19, 27). The ability of *S. pneumoniae* to ferment inulin was first discovered in 1905 and has been used as a diagnostic tool

to distinguish between pneumococci and other airway pathogens (13). Some other bacterial species are able to ferment inulin and shorter FOS. These include *Bifidobacteria* and *Lactobacilli* (8, 10). FOS transporters in other bacteria include PTS, ABC and major facilitator superfamily proteins (2, 9, 32). To date, the mechanism of growth of pneumococci on inulin, including the transporter, is unknown. We hypothesize that the proposed ABC sucrose transporter, SusT1T2X, is responsible for the transport of inulin and related FOS.

Methods

Bacterial Preparation and Growth Wild-type and genetically altered strains of *S. pneumoniae* and the recent clinical isolates used in this study are described in Table 1. Broth cultures were grown at 37°C in Todd-Hewitt broth (Becton, Dickenson, and Co., Sparks, MD) supplemented with 0.2% w/v yeast extract (Becton, Dickenson, and Co.) (THY). C media with 5% yeast extract (C+Y) pH 8 was used for transformations. *S. pneumoniae* was also grown at 37°C and 5 % CO_2 overnight on tryptic soy (TS) (Becton, Dickenson, and Co.) plates with 1.5% agar that were spread with 5000 U catalase (Worthington Biochemical Corporation, Lakewood, NJ) prior to plating bacteria and TS plates supplemented with 5% sheep blood (Becton, Dickinson, and Co.). Where appropriate, strains were selected for on TS plates that contained either streptomycin (200 µg/mL) or kanamycin (500 µg/mL) as appropriate. Unless otherwise stated, all carbohydrate solutions were sterilized using 0.22 µM Stericup (Millipore) filters. Unless otherwise indicated, all chemicals, substrates, and enzymes were purchased from Sigma Chemicals (St. Louis, MO).

Inulin Preparation using 1905 Protocol Inulin media was prepared as described by Hiss (13). Briefly, 33 mL of fetal bovine serum was added to 67 mL of water in a glass bottle. One gram of inulin was added and the mixture was stirred until the inulin was dissolved. The mixture was then placed in a beaker of water heated to 100ºC for 10 minutes. This heating was repeated on the following two days.

Genomic DNA Preparation All genomic DNA was prepared as previously described (36). Briefly, 10 mL of Optical Density at 600 nm (OD_{600}) = 0.8 culture was centrifuged and re-

suspended in 500 µL 50 mM ethylenediaminetetraacetic acid (EDTA)/ 10mM tris(hydroxymethyl)aminomethane (TRIS) (pH 8.0). Bacteria were incubated at 37°C with 20 µL 10 mg/mL lysozyme for 30 minutes (min) and subsequently with 30 µL 10 mg/mL proteinase K for an additional 30 min. 40 µL of 20 % v/v N-lauroyl sarcosine was added and mixed by inversion. After 10 min bacteria were subjected to two rounds of extraction with an equal volume of phenol/chloroform/isoamyl alcohol (Fisher Scientific, Pittsburgh, PA) and a final extraction with an equal volume of chloroform. DNA was isolated from the aqueous phase and precipitated with 100 % cold ethanol. The pellet was washed with 70 % v/v ethanol, vacuum dried, and re-suspended in 50 µL dH_2O.

Polymerase Chain Reaction All reactions were carried out in 0.2 mL flat cap PCR tubes. Master-mixes containing all reagents except the DNA template were pre-mixed and aliquoted into PCR tubes; DNA template was added individually to each reaction. H_2O rather than DNA template served as a negative control. When possible, positive controls were included.

Reactions using the high-fidelity polymerase, Phusion (New England Biolabs). A high fidelity polymerase was used to create of the desired construct for transformation into *S. pneumoniae*. To identify optimal amounts of DNA, neat, 1:5 and 1:10 dilutions of DNA were included until one was identified as most suitable. Primers were used at 0.4 µg/µL per manufacturer's instructions.

Reagent	*Amount (µL)*
5x *Phusion* PCR Buffer	5
50x dNTP (10 mM each)	1
Primer 1 (1 µg/µL)	0.2
Primer 2 (1 µg/µL)	0.2
Phusion polymerase	1
Chromosomal DNA template	0.3
De-ionized H_2O	To 50

Thermocycling Parameters in the BioRad MyCycler™ Thermal Cycler

95°C	Denature	30 s	1x
95°C	Denature	10 s	30x
≥60°C	Annealing	30 s	
72°C	Elongation	30 s /kb	
72°C	Polishing	10 min	1x
4°C			HOLD

The annealing temperature was determined for each reaction by estimating the temperature at which 50 % of the primer is dissociated (T_m) and using an annealing temperature 3°C less than that of the lowest primer if primer is twenty nucleotides or longer, if the primer is less than twenty nucleotides, the lower annealing temperature was used. Estimation of primer T_m was performed by counting each cytosine or guanine as 4°C and each adenine or thymine as 2°C.

Reactions using Choice™ Taq DNA Polymerase (Denville, Metuchen, NJ). When amplifying products for sequencing or screening potential mutants with primers flanking the intended recombination site, standard *Taq* polymerase was used. Standard 50 µL reaction:

Reagents	*Amount (µL)*
10x PCR Buffer	5
50x dNTP (10 mM each)	0.75
$MgCl_2$ (50mM)	0.5
Primer 1 (1 µg/µL)	0.2
Primer 2 (1 µg/µL)	0.2
Taq polymerase (5U/µL)	0.2
DNA template	0.3
De-ionized H_2O	To 50

Thermocycling Parameters in the BioRad MyCycler™ Thermal Cycler

95°C	Denature	4min	1x
95°C	Denature	1min	30x
55 ± 5°C	Annealing	1min	
72°C	Elongation	1min/kb	
72°C	Polishing	1min/kb	1x
4°C			HOLD

Electrophoresis of DNA on a 1% agarose gel Agarose gels were prepared by dissolving agarose in 1xTAE to 1% w/v. Ethidium bromide (final concentration = 0.025 µg/mL) was added directly to the melted agarose. To visualize chromosomal DNA or PCR products, 2 µL of DNA was mixed with 3 µL dH_2O and 2 µL loading dye. Samples were electrophoresed horizontally at 110 Volts in gels submerged in 1xTAE. DNA was visualized by UV-transillumination.

Sequence Analysis Before sequencing, PCR products were purified using the QIAquick PCR purification Kit (Qiagen). 50 ng/µl of purified PCR product was added to 4µl of the appropriate primer which was a concentration of 2µM. Sequencing reactions were done by Eurofins MWG Operon. DNA sequences of the genomic region flanking the gene(s) deleted of the mutant and parental sequences were compared using Lasergene SeqMan software (DNASTAR, Inc, Madison, WI). If a discrepancy was found between sequencing reactions, the chromatographic output was analyzed to determine the correct base. Sequence comparisons were performed using Lasergene MegAlign software. BLAST was used for protein sequence alignments and sequence identity.

Construction of Mutants All mutants were constructed using a Janus cassette selection system (34). This method requires two rounds of transformation. The first introduced a Janus cassette encoding kanamycin resistance and streptomycin sensitivity (*rpsL*$^{+}$*)* into the genome of streptomycin-resistant (Sm^{r}) *S. pneumoniae* strain in place of the region to be deleted. DNA fragments flanking the region to be deleted were amplified primers 1 and 2 (upstream region) and 4 and 5 (downstream region) and sequentially joined to the Janus cassette PCR product (primers J.F and J.R) using a variation of splicing by overlap extension (SOE) PCR (6), first described by Horton *et al.* (14). *Phusion* was used to minimize PCR-generated errors. The Janus construct was transformed into *S. pneumoniae* and the transformants were selected for on TS plates containing kanamycin and confirmed by PCR using primers 7 and 8, which flank the mutant construct.

The second round of transformation replaced the Janus cassette with an engineered segment of DNA consisting of the fragments flanking the deleted region. The fragments were generated using primers 1 and 3 (upstream fragment) and 5 and 6 (downstream fragment) and spliced together via SOE. The transformants were selected for on TS plates containing streptomycin. The unmarked mutants were confirmed with primers flanking the construct (primers 7 and 8) and sequencing. Mutants were grown on rich media to ensure no general growth defects were introduced during the mutant generation.

The genetically reconstituted strains were generated by transforming the final mutants with the corresponding Janus construct and then subsequently with parental DNA. Genetic reconstitution was confirmed by primers flanking the construct (7 and 8).

RNA Preparation and Reverse Transcriptase RT-PCR RNA was isolated by an acid-phenol extraction, with modifications for *S. pneumoniae* (16). Bacteria were grown as described for above for growth assays, but a final volume of 10 ml was incubated in a 37°C water bath. When samples reached OD_{600} of 0.3 ± 0.005, they were combined with 10 ml acid phenol and 100 µl 10% SDS and incubated at 90°C until the phases merged. Samples were then cooled on ice and centrifuged for 30 min at 3200 x g at 4°C. Two additional extractions were performed first with equal volumes of 1:1 acid phenol:choloroform and then with chloroform. RNA was precipitated with 10 ml isopropanol and 2 ml of 3 M sodium acetate at -20°C overnight. RNA was concentrated and washed twice with 1 ml 70% ethanol and then vacuum dried. Resulting RNA was resuspended in 100 µl H_2O, quantified, and adjusted to 100 µg 41 μl^{-1} to allow for a final reaction volume of 50 µl for DNase treatment. Nucleic acid was incubated with 5 µl DNase I buffer, 3 µl DNase I (New England Biolabs) and 1 µl Super RNaseIN (Promega) at 37°C for 1 hr. RNase-free reagents were used throughout. cDNA was generated with SmartScribe reverse transcriptase as according to the manufacturer's recommendations (New England Biolabs). Parallel samples were processed without addition of reverse transcriptase as a negative control.

The Janus system of mutant generation is designed to generate unmarked nonpolar mutants; however, as the genes encoding SusT1T2X are predicted to be upstream of and in the same transcriptional unit as *susH*, nonpolarity of the mutants were confirmed by semi-quantitative reverse-transcriptase PCR using primers 9 and 10 designed within the gene *susH*. The housekeeping gene, *aroE* (primers A.1 and A.2), was used as a control for measuring the level of gene expression. All primers used can be found in Table 2.

Growth Assays Chemically defined media (CDM) was prepared essentially as previously described (18), without the addition of carbohydrate. The CDM buffer was made at 2.5 times concentration to allow addition of sufficient carbohydrate to support bacterial growth. The medium was supplemented with no sugar, 12mM glucose, 12mM sucrose, 5mg mL^{-1} inulin, 12 mM kestose (Wako Chemicals), 12 mM nystose (Wako Chemicals), 12 mM fructofranosylnystose (Wako Chemicals),or 2.7 mg ml^{-1} raftilose (Beneo-Orafti). *S. pneumoniae* strains were grown in THY to an optical density at 600 nm (OD_{600}) = 0.3 ± 0.005, 1 mL aliquots were washed and resuspended in 65 µl of PBS and 65 µl of catalase (50,000 U mL^{-1}). Twenty microliter aliquots of bacterial suspensions or PBS/catalase (no bacteria control) were transferred to 180 µl of CDM supplemented with the appropriate carbon source. Medium supplemented with glucose served as a positive control, indicating the bacterial were viable and that mutant strains showed no general growth defect relative to their parent strain. Medium with no added carbohydrate served as a negative control. Growth plates were incubated at 37°C for 60 h in a BIO-TEK Synergy HT plate reader and the OD_{600} was read every 20 min. All data were adjusted to a path-length of 1 cm. As no increase in optical density was observed for any bacterial strain on no-carbohydrate medium, at each time point the average of results from triplicate wells was subtracted from results from all other wells containing the same bacterial strain. Results from triplicate wells were then averaged. Growth experiments were performed three times in triplicate with the exception of the growth of clinical isolates on nystose and inulin. Clinical strains that showed no growth on these carbohydrates were tested at least one additional time. While each separate experiment showed the same trends, the growth profile varied so the graphs shown are representative curves.

Table 1: Strains used in this study.

Strain Name	Serotype	Characteristics/genotype[a]	Source or Reference
TIGR4	4	Clinical isolate	(35)
TIGR4 Sm^r	4	Lys→Thr in RpsL [*rpsL*(*K56T*)] conferring Sm^r	(3)
TIGR4 Δ*msmK*	4	Δ*msmK rpsL*(*K56T*) (Sm^r)	This study
TIGR4 Δ*msmK/msmK*$^+$	4	Lys→Thr in RpsL [*rpsL*(*K56T*)] conferring Sm^r	This study
TIGR4 Δ*susT1T2X*	4	Δ*sus rpsL*(*K56T*) (Sm^r)	This study
TIGR4 Δ*susT1T2X/susT1T2X*$^+$	4	Lys→Thr in RpsL [*rpsL*(*K56T*)] conferring Sm^r	This study
TIGR4 Δ*scrT*	4	Δ*scrT rpsL*(*K56T*) (Sm^r)	This study
TIGR4 Δ*scrT/scrT*$^+$	4	Lys→Thr in RpsL [*rpsL*(*K56T*)] conferring Sm^r	This study
TIGR4 Δ*scrT*Δ*susT1T2X*	4	Δ*scrT* Δ*sus rpsL*(*K56T*) (Sm^r)	This study
TIGR4 Δ*scrT*Δ*susT1T2X/scrT*$^+$*susT1T2X*$^+$	4	Lys→Thr in RpsL [*rpsL*(*K56T*)] conferring Sm^r	This study
C06_4	6A/B	Clinical isolate	This study
C06_5	15A	Clinical isolate	(24)
C06_6	19A	Clinical isolate	This study
C06_8	19A	Clinical isolate	This study
C06_9	19A	Clinical isolate	This study
C06_10	3	Clinical isolate	(24)
C06_12	19A	Clinical isolate	This study
C06_14	19A	Clinical isolate	(24)
C06_18	22F	Clinical isolate	(6)
C06_19	6A/B	Clinical isolate	This study
C06_20	19A	Clinical isolate	This study
C06_21	19A	Clinical isolate	This study
C06_23	19A	Clinical isolate	This study
C06_25	3	Clinical isolate	This study
C06_31	23F	Clinical isolate	(6)
C06_34	35F	Clinical isolate	This study
C06_36	19A	Clinical isolate	This study
C06_39	35F	Clinical isolate	(6)
C06_40	19A	Clinical isolate	This study
C06_44	19A	Clinical isolate	This study
C06_45	19A	Clinical isolate	This study
C06_48	19A	Clinical isolate	This study
C06_50	19A	Clinical isolate	This study
C06_57	6/AB	Clinical isolate	(6)
C06_60	19F	Clinical isolate	This study
CI41	19	Clinical isolate	(28)
Cr3	4	Clinical isolate	(28)
CI7	6B	Clinical isolate	(28)

Table 1: Strains used in this study (continued).

Strain Name	Serotype	Characteristics/genotype[a]	Source or Reference
Cr31	11	Clinical isolate	(28)
Cl50	23F	Clinical isolate	(28)
Cl32	15	Clinical isolate	(28)
Cl43	19	Clinical isolate	(28)
Cl15	9	Clinical isolate	(28)
Cl25	12	Clinical isolate	(28)

[a] Sm^r indicates resistance to Streptomycin

Table 2: Primers used in this study.

Group	No.	Primer Sequence (5' → 3')[a]	Location (accession no.)[b]
Janus	J.F	CCGTTTGATTTTTAATGGATAATG	7-30 (AY334019)
	J.R	GGGCCCCTTTCCTTATGCTT	247511-247527 (AE005672)
aroE	A.1	GCCTTTGAGGCGACAGC	1299642-1299626 (AE005672)
	A.2	TGCAGTTCARAAACATWTTCTAA	1299164-1299186 (AE005672)
susT1T2X	U.1	GCAGGTGGAAGAGAAGCAG	1715812-1715830 (AE005672)
	U.2	CATTATCCATTAAAAATCAAACGGGGACAGTCATCAATAACAAGAG (1)	1715089-1715110 (AE005672)
	U.3	ATCATCCCACTCAGTATCAAGGACAGTCATCAATAACAAGAG (3)	1715089-1715110 (AE005672)
	U.4	AAGCATAAGGAAAGGGGCCCTTGATACTGAGTGGGATGAT (2)	1711791-1711810 (AE005672)
	U.5	CCTAGTAGAACGATACAGCC	1711791-1711810 (AE005672)
	U.6	TTGATACTGAGTGGGATGAT	1711111-1711130 (AE005672)
	U.7	TGCGTCATAATAAAGTTGATGG	1715949-1715970 (AE005672)
	U.8	ATCTAACTCGAAGTAATCTGGG	1711011-1711032 (AE005672)
	U.9	CAGGCTCTGCCATTGTCA	1711373-1711390 (AE005672)
	U.10	TCCCTCACGCTGATAA	1710963-1710978 (AE005672)
scrT	C.1	CTCAACATGGTAATGAGTATGC	1627457-1627478 (AE005672)
	C.2	CATTATCCATTAAAAATCAAACGGTTCGCCCTTACGACTATTAT (1)	1627038-1627057 (AE005672)
	C.3	CACATTAGAGAGGATTGATTAGATTCGCCCTTACGACTATTAT (4)	1627038-1627057 (AE005672)
	C.4	AAGCATAAGGAAAGGGGCCCTCTAATCAATCCTCTCTAATGTG (2)	1625085-1625107 (AE005672)
	C.5	GACCAGCTGCATAACCTTCTA	1625085-1625107 (AE005672)
	C.6	TCTAATCAATCCTCTCTAATGTG	1624384-1624404 (AE005672)
	C.7	GGGTCGTTGAGAAGTCCTGTT	1627484-1627504 (AE005672)
	C.8	ACCTGTACGAGCTTCCAAACT	1624361-1624381 (AE005672)
	C.9	CAATTTCCAACAACAACTCC	1624845-1624864 (AE005672)
	C.10	CATAACCTTCTAGACATCCC	1624393-1624412 (AE005672)
msmK	M.1	TAGACAAAAGCAGAAACAAGGATC	1486427-1486450 (AE005672)
	M.2	CATTATCCATTAAAAATCAAACGGATGTCTTCTTTGCTGTATTTACGC (1)	1485931-1485954 (AE005672)
	M.3	CAAGTCAAATCCAAGCTCAACATGTCTTCTTTGCTGTATTTACGC (6)	1485931-1485954 (AE005672)
	M.4	GGAAAGGGGCCCAGGTGGTTGAGCTTGGATTTGACTTG (2)	1485183-1485203 (AE005672)
	M.5	GTTGAGCTTGGATTTGACTTG	1485183-1485203 (AE005672)
	M.6	TGACCTGCTTCTGACATTTGA	1484618-1484638 (AE005672)
	M.7	CTACACAAAAATAAGCTCCATAAT	1486508-1486531 (AE005672)
	M.8	TTCCCCTATTACACGCAACCT	1484490-1484510 (AE005672)

[a] Underlining indicates reverse complement sequence of primer J.F (1) J.R (2) U.6 (3) C.6 (4) H.6 (5) and M.6 (6).

[b] Locations are given as nucleotide positions in the indicated accession numbers.

Results

***susT1T2X* is required for growth on FOS** To determine if *susT1T2X* contributes to FOS utilization in the *S. pneumoniae* strain TIGR4 Sm^r, an unmarked nonpolar deletion of the three transporter genes was generated. Growth on commercially available short chain FOS with the same linkages as inulin, glucose linked to two fructose (kestose or GF2), three fructose (nystose or GF3), or four fructose (fructofranosylnystose or GF4), oligofructose consisting of two to eight fructose, (raftilose) and inulin was tested. TIGR4 Sm^r was able to grow on all FOS tested, yet growth on GF2 ($OD_{600nm} \geq 0.121$) and oligofructose ($OD_{600nm} \geq 0.28$) was consistently less robust than growth on inulin ($OD_{600nm} = 0.25$ to 0.55), GF3 and GF4 ($OD_{600nm} \geq 0.60$). TIGR4 Δ*susT1T2X* was unable to grow on GF3 and longer FOS (Fig 3).

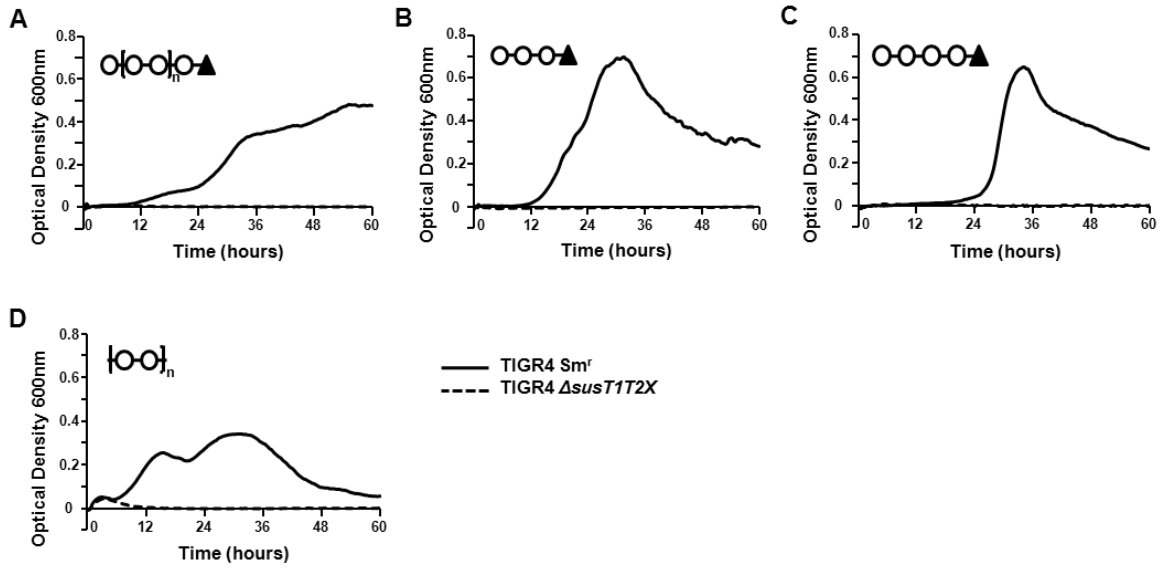

Figure 3. *susT1T2X* is required for growth on GF3, GF4, inulin and oligofructose. TIGR4 Sm^r and TIGR4 Δ*susT1T2X* were grown for 60 h on chemically defined medium (CDM) supplemented with (A) 5 mg ml^{-1} inulin, (B) 12mM GF3, (C) 12 mM GF4, or (D) 2.7 mg ml^{-1} oligofructose as the sole carbon source. Growth was measured by the optical density at 600 nm. A CDM no sugar control has been subtracted from each data set. Each experiment was performed at least three independent times in triplicate and the OD_{600} values represent one experiment. On the graphs, circles represent fructose molecules and triangles represent glucose molecules.

TIGR4 Δ*susT1T2X* grew comparably to TIGR4 Sm^r on glucose and a genetically reconstituted strain TIGR4 Δ*susT1T2X/susT1T2X*$^+$ grew comparably to TIGR4 Sm^r on each carbohydrate tested indicating the phenotype seen with TIGR4 Δ*susT1T2X* is due only to the absence of the transporter genes (data not shown). Growth of TIGR4 Δ*susT1T2X* was not reduced on GF2 indicating GF2 transport is not *sus* dependent. Growth on GF2 is partly due to the PTS sucrose transporter, ScrT, as a growth of TIGR4 Δ*scrT* is reduced, but not eliminated, compared to the parental strain on GF2 (Fig. 4). A double *susT1T2X/scrT* mutant showed no further reduction over the *scrT* mutant indicating the residual growth seen with the *scrT* mutant is not due to *susT1T2X*.

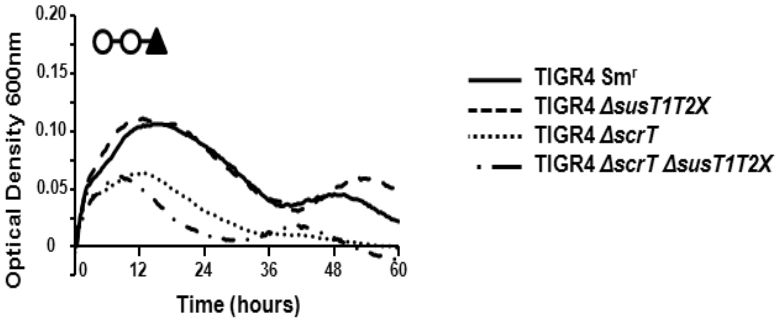

Figure 4. Growth on GF2 involves multiple transporters. TIGR4 Sm^r, TIGR4 Δ*susT1T2X*, TIGR4 Δ*scrT* and TIGR4 Δ*scrT* Δ*susT1T2X* were grown for 60 h on chemically defined medium (CDM) supplemented with 12 mM GF2 as the sole carbon source. Growth was measured by the optical density at 600 nm. A CDM no sugar control has been subtracted from each data set. This experiment was performed at least three independent times in triplicate and the OD_{600} values represent one experiment.

***msmK* is required for growth on FOS** SusT1T2X is one of six CUT1 transporters encoded in the TIGR4 genome (35). All six of the transporters lack a gene encoding an ATPase in their genomic region, an essential component of the transporters (35). The gene *msmK* is predicted to encode for the ATPase that energizes all six carbohydrate ABC transporters (21). The substrates of three of the six transporters have been previously identified and MsmK has been shown to be

essential for growth on those three carbohydrates (1, 21, 22, 30). Growth of TIGR4 Δ*msmK* on GF3 was completely eliminated (maximal $OD_{600nm} \leq 0.014$) (Fig. 5) while TIGR4 Δ*msmK* grew normally on glucose and TIGR4 Δ*msmK*/Δ*msmK*$^{+}$ grew comparably to TIGR4 Sm^{r} on GF3 (maximal $OD_{600nm} \geq 0.52$). These data indicate SusT1T2X is also energized by MsmK.

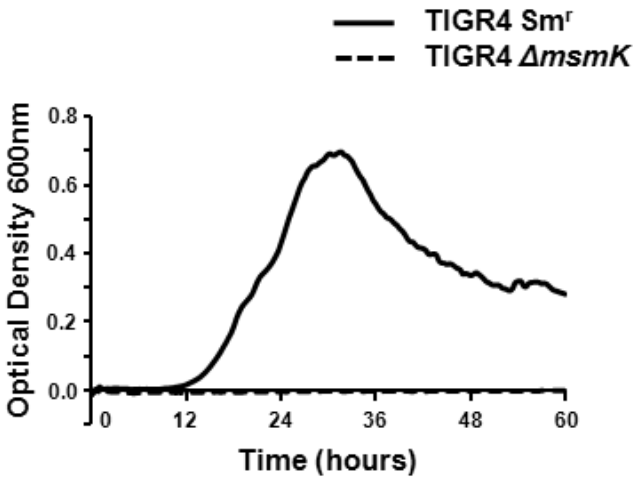

Figure 5. *msmK* is required for growth on GF3. Growth of *S. pneumoniae* strain TIGR4 and TIGR4 Δ*msmK* on chemically defined media (CDM) supplemented with 12 mM GF3 as the sole carbon source. A CDM no sugar control has been subtracted from each data set. The growth assay was performed three independent times in triplicate and the OD_{600} values represent one experiment.

Media preparation affects pneumococcal growth on inulin It has been reported that there is strain-to-strain variation in the ability of pneumococci to utilize inulin for growth and while TIGR4 Sm^{r} was able to grow on inulin, another common lab strain 1121 Sm^{r} was unable to grow on inulin each time tested. The ability to ferment inulin was used as a diagnostic test for pneumococci yet not every strain can utilize inulin for growth. In 1905, to sterilize the inulin media, the media was heated at 100°C for ten minutes for three consecutive days (13). We proposed during this heating process some of the long fructose chains were broken down and it was these shorter fragments that the bacteria were using for growth. This hypothesis is supported by the fact that TIGR4 Sm^{r} and 1121 Sm^{r} were both able to grow on inulin media that had been

autoclaved (20 min, 120ºC, 17 PSI), not filter sterilized as was done with the rest of the carbohydrates (1121 Sm^r maximal $OD_{600nm} \geq 0.20$, TIGR4 Sm^r $OD_{600nm} \geq 0.44$) (Fig. 6).

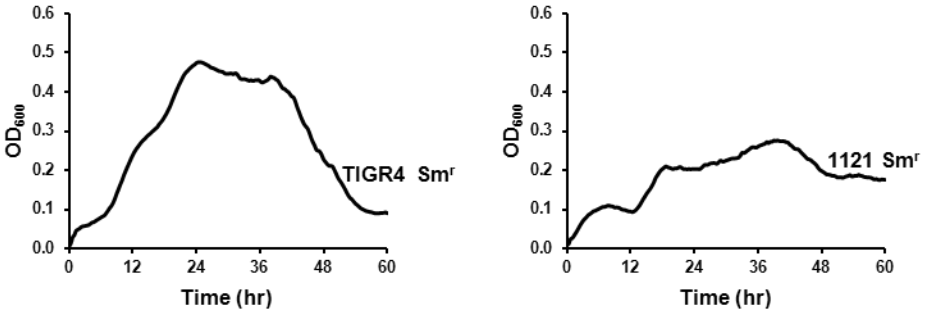

Figure 6. TIGR4 Sm^r and 1121 Sm^r can grow on autoclaved inulin. Growth of *S. pneumoniae* strain TIGR4 and 1121 on chemically defined media (CDM) supplemented with 5 mg ml^{-1} autoclaved inulin as the sole carbon source. A CDM no sugar control has been subtracted from each data set. The growth assay was performed three independent times in triplicate and the OD_{600} values represent one experiment.

Growth of TIGR4 Δ*susT1T2X* was completely eliminated on the autoclaved inulin indicating the growth seen was not due to free fructose, glucose or sucrose in the media (Fig. 7).

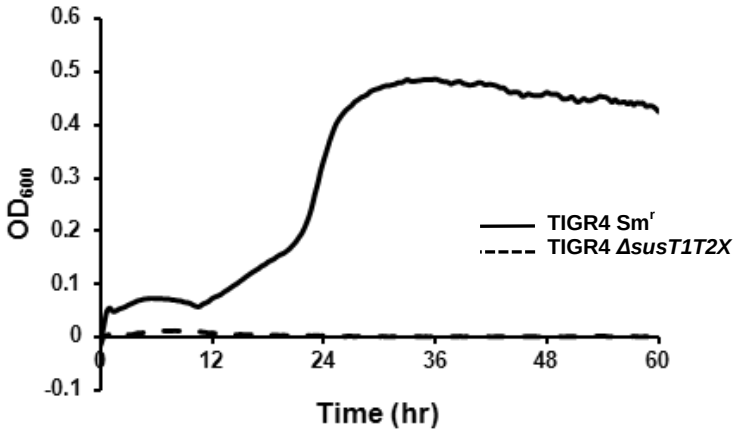

Figure 7. *susT1T2X* is required for growth on autoclaved inulin. Growth of *S. pneumoniae* strain TIGR4 and TIGR4 Δ*susT1T2X* on chemically defined media (CDM) supplemented with 5 mg ml^{-1} autoclaved inulin as the sole carbon source. A CDM no sugar control has been subtracted from each data set. The growth assay was performed three independent times in triplicate and the OD_{600} values represent one experiment.

TIGR4 Δ*susT1T2X/susT1T2X*$^+$ grew comparably to TIGR4 Sm^r on the autoclaved inulin (data not shown).

As the autoclaving process most likely partially degraded the long fructose chains, this supports the hypothesis that short chain FOS support pneumococcal growth. The growth profile of 1121 Sm^r indicates that while long-chain FOS (inulin) cannot support 1121 growth, short-chain FOS can. We tried multiple times to recreate the inulin media used in 1905. TIGR4 Sm^r and 1121 Sm^r were able to grow significantly on the 1905 prepared inulin yet so was TIGR4 Δ*susT1T2X* indicating the heating process caused too much degradation of the fructose chains (data not shown).

Differences in the ability of pneumococcal strains to utilize inulin correlates with the transporter encoded at this genomic locus It has been reported the *sus* locus is not a core part of the pneumococcal genome (15, 29). While the predicted sucrose hydrolase, SusH, and the predicted LacI family repressor, SusR, are encoded by all sequenced strains, in some strains an alternate carbohydrate ABC transporter is encoded at the *sus* locus (Fig 8).

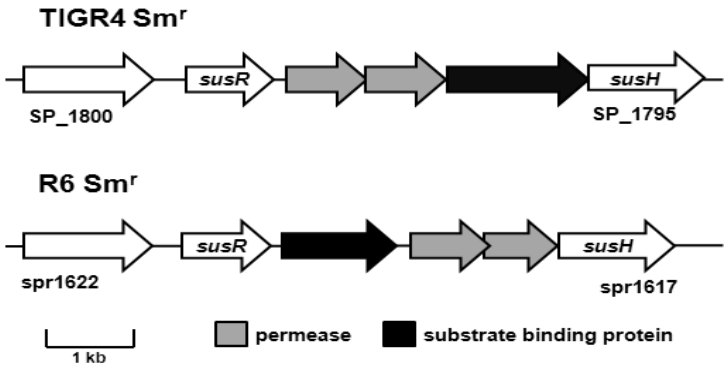

Figure 8. Schematic of putative FOS ABC transporters in *S. pneumoniae* strains TIGR4 and R6. 1121 is not sequenced so R6 is used as a representative. The substrate binding proteins are in black and the membrane spanning permeases are gray. A *lacI* family repressor *susR* and a sucrose hydrolase *susH* are conserved across pneumococcal strains. Also conserved is the first gene in each proposed operon, which is predicted to be a transcriptional activator.

The two transporters share little sequence similarity in their predicated amino acid sequences. The substrate binding proteins essentially share no sequence similarity, while the two permease components share 27% and 30% amino acid identity over the full length of the predicted amino acid sequence. The *sus* locus is present in 104 of the 123 pneumococcal genomes available and the alternative carbohydrate ABC transporter is present in the remaining 19.

While we currently do not have the sequence of 1121 Sm^r, through PCR we were able to determine the alternate transporter is encoded at the *sus* locus. As TIGR4 and 1121 encode different transporters at the *sus* locus and we showed these strains differ in their ability to utilize long chain FOS, we investigated if the different transporters were responsible for the different phenotypes. Thirty-four recent clinical isolates representing 13 serotypes were PCR screened to determine which transporter was encoded at the *sus* locus. This was done using a common primer designed in the gene encoding the sucrose hydrolase and one of two unique primers designed in the gene encoding the substrate binding protein of *sus* and a gene encoding one of the permease proteins of the alternate transporter (*sus*-like). These 34 strains were also tested for growth on short chain (GF3) and long chain (inulin) FOS (Table 3). Thirty-two of the 34 strains were able to grow on GF3 (maximal OD_{600nm} ranging from 0.34 to 0.97) and 27 of the 34 strains were able to grow on inulin (no growth was defined as $OD_{600nm} \leq 0.02$). All of the clinical isolates that were able to grow on inulin encode the same carbohydrate ABC transporter at the *sus* locus as TIGR4. Of the seven strains that did not grow on inulin, six of them encode the alternate transporter. One of the two strains that did not grow on GF3 encodes the alternate transporter and grew poorly on glucose compared to other strains indicating this strain may not grow well under laboratory conditions. One strain that did not grow on inulin encodes the same

transporter as TIGR4 yet is one of the strains that did not grow on GF3 indicating a protein(s) required for FOS utilization may not be functional. Currently, we do not have sequences of either strain that did not grow on GF3, so it is possible there is a mutation in a gene(s) required for FOS utilization. These data indicate both transporters encoded at the *sus* locus allow for import of short chain FOS but only the *sus* transporter allows for import of long chain FOS.

Table 3. Growth of Clinical Isolates on GF3 and inulin

Strain	Growth[a]		Allele
	GF3	inulin	
C06_4	+	+	*sus*
C06_5	+	-	*sus*-like
C06_6	+	+	*sus*
C06_8	+	+	*sus*
C06_9	-	-	*sus*-like
C06_10	+	+	*sus*
C06_12	+	+	*sus*
C06_14	+	+	*sus*
C06_18	+	+	*sus*
C06_19	+	-	*sus*-like
C06_20	+	+	*sus*
C06_21	+	-	*sus*-like
C06_23	+	+	*sus*
C06_25	+	+	*sus*
C06_31	+	-	*sus*-like
C06_34	+	+	*sus*
C06_36	+	+	*sus*
C06_39	+	+	*sus*
C06_40	+	+	*sus*
C06_44	+	+	*sus*
C06_45	+	+	*sus*
C06_48	+	+	*sus*
C06_50	+	+	*sus*
C06_57	+	-	*sus*-like
C06_60	+	+	*sus*
Cl41	+	+	*sus*
Cr3	+	+	*sus*
Cl7	+	+	*sus*
Cr31	+	+	*sus*
Cl50	+	+	*sus*
Cl32	+	+	*sus*
Cl43	+	-	*sus*-like
Cl15	+	+	*sus*
Cl25	+	+	*sus*

[a]No growth is defined as not reaching an OD_{600} higher than 0.02

Discussion

It has been previously demonstrated that SusT1T2X contributes to sucrose transport in vitro (15). However, we showed that a mutant in *susT1T2X* does not affect growth on sucrose and a double mutant in the sucrose PTS, *scrT*, and *susT1T2X* is no further reduced in growth over the single PTS mutant. This evidence suggests ScrT is the major sucrose transporter and that the main substrate of SusT1T2X is another carbohydrate.

Inulin is known to be fermented by about 60% of pneumococcal strains yet no transporter had been identified (19, 27). The findings in the study show that *S. pneumoniae* can utilize multiple lengths of FOS for growth. Growth of a TIGR4 *susT1T2X* mutant was completely eliminated on all FOS tested longer than GF2, including inulin, supporting the hypothesis that SusT1T2X acts as a FOS and not a sucrose transporter.

The TIGR4 *susT1T2X* mutant showed no reduction in growth on GF2 compared to the parent strain. The data show GF2 is transported by multiple transporters as an *scrT* mutant was reduced but not eliminated in growth compared to the parent strain. A double *scrT/susT1T2X* mutant showed no additional reduction compared to the single *scrT* mutant indicating the residual growth seen with the *scrT* mutant is not due to SusT1T2X. Currently we do not know what other transporter(s) are contributing to growth on GF2. Raffinose is a sucrose containing carbohydrate and is known to be fermented by pneumococci so it is possible the raffinose transporter, encoded by *rafEFG* (SP1895-7), is contributing to growth on GF2 (30).

We have demonstrated the ATPase MsmK is required for growth on GF3. SusT1T2X is one of six CUT1 family transporters encoded by TIGR4, all of which lack a gene encoding an ATPase in their genomic region. SusT1T2X is the fourth CUT1 family transporter to be shown to require MsmK to be functional providing more evidence to support the hypothesis that MsmK energizes all six CUT1 family transporters. It was recently reported MsmK was not required for growth on sucrose and this was used as evidence to suggest MsmK does not energize SusT1T2X (4). As we believe the main sucrose transporter is part of the PTS, we would predict MsmK would play no role in sucrose transport.

While it is known that some bacteria degrade FOS outside the cell, no extracellular enzyme has been identified in *S. pneumoniae* that would be involved in the breakdown of FOS before transport (35). It is currently unknown what lengths of inulin pneumococci are actually importing. By performing mass spectrometry on media before and after bacterial incubation, we may be able to determine exactly what chain lengths of inulin are being utilized by *S. pneumoniae.* Also, structuring the substrate binding proteins of the *sus* transporter and the alternate transporter would provide information about the binding pocket and may help determine which lengths of FOS are binding to each protein. This may help determine whether the substrate binding protein or permeases are responsible for the differences in specificity between the two transporters at the *sus* locus.

While inulin was reported to be fermented by pneumococci in 1905, it has also been reported that not every strain can ferment inulin (4, 13, 19, 27). It has also been reported the *sus* locus is not a core part of the pneumococcal genome; in some strains there is an alternate carbohydrate

ABC transporter encoded at that locus (15, 29). Both transporters appear to be FOS transporters yet we have had difficulty in making a mutant of the alternate transporter. An *msmK* mutant in a strain encoding the alternate ABC transporter is unable to grow on GF3, indicating transport is through an ABC transporter and the best candidate is the one encoded at the *sus* locus. It is interesting that both transporters are being maintained in the population. While it is possible the two transporters are important during different phases of pathogenesis, so far we have seen no evidence of a correlation between site of isolation and the transporter encoded. As both transporters appear to allow for the import of short chain FOS, it seems more likely that the biologically relevant substrate is transported equally well by both transporters.

It has previously been reported that *sus* locus contributes to in vivo fitness in the lung and not the nasopharynx. It is unlikely FOS are present in the lung but it is possible the transporter has other substrates that are. Inulin and other FOS are not synthesized by humans and are typically considered dietary carbohydrates. There is evidence that pneumococci can be found in the mouth yet the number and frequency of this is unknown. There are a number of dietary carbohydrates known to be fermented by pneumococci indicating the bacteria may see them during some stage of pathogenesis (4, 25, 26, 33). Another possible source of FOS in vivo is from fructans made by other bacteria. There is evidence that some bacteria species, including *S. mutans*, express extracellular polysaccharide consisting of fructans with the same structure as oligofructose (5, 7). This suggests that bacteria in the same niche as the pneumococcus may produce fructans that can be utilized by the pneumococcus for growth. Previous studies have suggested that pneumococci can utilize capsular carbohydrates produced by other bacteria for growth; for example the

Streptococcus pyogenes hyaluronic acid capsule can be used by pneumococci a sole carbon source (24).

Sequence homology searches revealed that the genes encoding the alternative transporter are closely related to a predicted ABC transporter encoded by *Streptococcus mitis*. Comparison of the sequence of D39 which encodes the alternative ABC transporter and *S. mitis* strain NCTC 12261 revealed 98.5% identity between the nucleotide sequences and 99% identity between the predicted amino acid sequences of the substrate binding proteins. Furthermore, each permease component encoded by D39 shares greater than 98.8% identity at both the nucleotide and amino acid levels with the putative permeases encoded by NCTC 12261. These data suggest that the alternative locus may have been introduced into pneumococci through homologous recombination with *S. mitis.*

In summary, the data presented here demonstrate the mechanism by which pneumococci utilize FOS and determines the likely reason for the previously identified variation in the ability of pneumococci to utilize inulin.

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