Sucrose metabolism contributes to *in vivo* fitness of *Streptococcus pneumoniae*

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Summary

We characterized two sucrose-metabolizing systems – *sus* and *scr* – and describe their roles in the physiology and virulence of *Streptococcus pneumoniae* in murine models of carriage and pneumonia. The *sus* and *scr* systems are regulated by LacI family repressors SusR and ScrR respectively. SusR regulates an adjacent ABC transporter (*susT1/susT2/susX*) and sucrose-6-phosphate (S-6-P) hydrolase (*susH*). ScrR controls an adjacent PTS transporter (*scrT*), fructokinase (*scrK*) and second S-6-P hydrolase (*scrH*). *sus* and *scr* play niche-specific roles in virulence. The *susH* and *sus* locus mutants are attenuated in the lung, but dispensable in nasopharyngeal carriage. Conversely, the *scrH* and *scr* locus mutants, while dispensable in the lung, are attenuated for nasopharyngeal colonization. The *scrH/susH* double mutant is more attenuated than *scrH* in the nasopharynx, indicating SusH can substitute in this niche. Both systems are sucrose-inducible, with ScrH being the major *in vitro* hydrolase. The *scrH/susH* mutant does not grow on sucrose indicating that *sus* and *scr* are the only sucrose-metabolizing systems in *S. pneumoniae*. We propose a model describing hierarchical regulation of the *scr* and *sus* systems by the putative inducer, S-6-P. The transport and metabolism of sucrose or a related disaccharide thus contributes to *S. pneumoniae* colonization and disease.

Introduction

Carbon acquisition and metabolism are central to life. The regulation of these processes in bacteria and eukaryotes is complex and necessarily involves timely and appropriate responses to changes in nutrient availability to maintain optimal cellular growth rates (Titgemeyer and Hillen, 2002). Adequate control over processes involved in nutrient acquisition and metabolism are likely to be central to host niche dominance and fitness of microbial pathogens during carriage and infection. The role of carbon metabolism and its relevance in determining *in vivo* fitness, while intuitively important, has only recently begun to be appreciated (Smith, 2000; Chico-Calero *et al.*, 2002; Munoz-Elias and McKinney, 2006). Recently studied examples include carbon metabolic pathways such as gluconeogenesis and the TCA cycle (Tchawa Yimga *et al.*, 2006), the Entner–Doudoroff pathway (Sweeney *et al.*, 1996a,b;

Supplementary material

This material is available as part of the online article from:

<http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2007.05878.x>

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Peekhaus and Conway, 1998), and specific carbon utilization genes (Chang *et al.*, 2004) in the enterics, *Escherichia coli* and *Salmonella* sp. In addition to sugars, metabolism of fatty acids has also recently been shown to be required for the virulence and persistence of the lung pathogen, *Mycobacterium tuberculosis* (McKinney *et al.*, 2000; Munoz-Elias and McKinney, 2005) and has revealed novel and unexpected bacterial enzyme targets for future drug therapies.

Streptococcus pneumoniae is a Gram-positive encapsulated bacterium that is the leading cause of bacterial meningitis, pneumonia and otitis media (McCullers and Tuomanen, 2001; Paton and Giammarinaro, 2001; Hava *et al.*, 2003a; Bogaert *et al.*, 2004). While the study of *S. pneumoniae* pathogenesis has focused on factors involved in host attachment, damage and immune evasion (Gosink *et al.*, 2000; Hava *et al.*, 2003b; Johnston *et al.*, 2004; Orihuela *et al.*, 2004; Shaper *et al.*, 2004; Barocchi *et al.*, 2006), scant information is available on metabolic pathways and specific carbon sources used during colonization and infection within the host. Signature-tagged mutagenesis screens designed to identify genes with roles in colonization and infection have revealed mutations in several systems involved in carbon metabolism. Many of these insertions localized to carbohydrate uptake and catabolic systems, including both ATPase-Binding Cassette (ABC) transporters and Phosphotransferase Systems (PTS) (Polissi *et al.*, 1998; Lau *et al.*, 2001; Hava and Camilli, 2002) underscoring the potential involvement of these genes in *in vivo* fitness.

The LacI/GalR family are well-studied transcriptional repressors that typically mediate inducible regulation of operons involved in carbon metabolism (Weickert and Adhya, 1992). One example of this family, CcpA (Catabolite control protein A), first discovered as a repressor of the *Bacillus subtilis* amylase operon (Henkin *et al.*, 1991), has since been shown to be vital to the co-ordinated expression of a variety of carbon utilization operons, including the processes of carbon catabolite repression and activation in Gram-positive bacteria (Luesink *et al.*, 1998; Stulke and Hillen, 2000; Kim *et al.*, 2002; Viana *et al.*, 2005) including streptococci (Simpson and Russell, 1998; Rogers and Scannapieco, 2001; Asanuma and Hino, 2006). In previous studies, it was established that the *S. pneumoniae* CcpA orthologue contributes to the ability of this pathogen to colonize the mouse nasopharynx and infect the lung (Iyer *et al.*, 2005) and bloodstream (Iyer *et al.*, 2005). Although the molecular underpinnings of the *ccpA* defect in colonization and disease are unclear, the attenuation seen for the *ccpA* strain *in vivo* highlights the role of carbon source regulation in *in vivo* fitness in *S. pneumoniae*. This prompted us to investigate carbohydrate metabolic systems that are specifically induced during infection and explore the contribution of other LacI/GalR members to the regulation of such systems. There are five additional LacI/GalR family members in *S. pneumoniae*, one of which, RegR, has already been shown to contribute to virulence (Chapuy-Regaud *et al.*, 2003). Two genes encoding other members of this family are proximal to and likely to regulate two putative sucrose transport systems. We investigated if the ability to transport and metabolize sucrose contributes to *in vivo* fitness of this pathogen. Here we show that sucrose uptake and catabolism play important roles in colonization of the murine nasopharynx and an accessory role in the infection of the lung, but is dispensable in a model of bacteraemia. Our data also indicate that the two sucrose uptake systems are differentially expressed such that one is dominant during lung infection while the other is dominant in colonization of the nasopharynx. Thus, the disaccharide sucrose is a major carbon source utilized by *S. pneumoniae* during colonization and infection of mucosal surfaces.

Results

The *sus* locus and the putative sucrose phosphate hydrolase, *SusH*, contribute to *in vivo* fitness in lung

SP1799, herein named *SusR* for sucrose utalization system repressor, is a LacI family member that lies adjacent to a putative ABC transporter locus, containing the solute-binding lipoprotein

SusX, transmembrane components SusT1 and SusT2, and a cytoplasmic sucrose-6-phosphate (S-6-P) hydrolase, SusH. Our prediction that this locus mediates sucrose metabolism was based on our observation that both SusH and its paralogue (ScrH, see below) contain a signature β -fructosidase motif (NDPNG) (Ritsema *et al.*, 2004) which is conserved across bacteria, plants and fungi. To test if this locus contributes to *in vivo* fitness, we deleted the entire set of genes leaving behind a chloramphenicol (Cm) resistance cassette (*cat*). The deletion of this locus leads to a threefold decrease in bacterial load in the lung in a competition with wild type (Fig. 1). Consistent with a concerted role for this locus in transport of sucrose, strains either lacking the membrane components, *susX/susT1/susT2* (not shown), or just the putative S-6-P hydrolase, *susH*, are mildly attenuated in the lung (Fig. 1).

The SusH paralogue, SP1724, herein named ScrH for sucrose hydrolase, lies divergent from another LacI family member, SP1725 (ScrR), which is part of a putative sucrose-specific PTS. This putative operon contains the PTS components SP1722 (ScrT) and fructokinase, SP1724 (ScrK). We hypothesized that the *sus* and *scr* systems might be partially redundant for sucrose uptake during infection and we tested this by comparing the *in vivo* fitness of each single- and double-hydrolase null, *susH/scrH*. While the *scrH* mutant is only slightly attenuated in the mouse lung (Fig. 1), there is no further attenuation for the double-hydrolase mutant, compared with the *susH* mutant alone (Fig. 1). A similar level of attenuation was observed for a strain missing both sucrose operons, *sus/scr* (Fig. 1). Provision of *susH* *in trans* completely rescues the *scrH/susH* strain defect in the lung, showing that the loss of the ABC transporter-associated sucrose hydrolase, SusH, contributes to fitness in the lung. Both S-6-P hydrolases are dispensable for survival in a murine model of bacteraemia (data not shown).

The S-6-P hydrolase ScrH contributes to colonization of the murine nasopharynx

Colonization of the nasopharynx is an important step in the normal life cycle of *S. pneumoniae* and is thought to precede infection of other sites in diseased patients (Kadioglu *et al.*, 2002; Kwon *et al.*, 2004; Ogunniyi *et al.*, 2007). To assess the contribution of sucrose metabolism to nasopharyngeal colonization, we performed competition assays on the two single- and double-hydrolase mutants. Our results are converse to those in the mouse lung (above) in that the *susH* strain remains virulent while the *scrH* null is out-competed threefold in the nasopharynx (Fig. 2). The double mutant is, however, out-competed ~30-fold in the nasopharynx, indicating that SusH is able to substitute at least partially for ScrH function (Fig. 2). Additionally, provision of *scrH* *in trans* along with 360 bp of native upstream sequence which should contain its promoter (referred to as *NscrH*; see *Experimental procedures*) completely rescued the colonization defect of the *susH/scrH* mutant (Fig. 2) showing that the S-6-P hydrolase, ScrH, contributes to fitness in the murine nasopharynx. Our data show that the PTS-associated S-6-P hydrolase, *scrH*, is the main hydrolase required for colonization fitness in the nasopharynx whereas the ABC-associated S-6-P hydrolase is dominant in the mouse lung infection model. Moreover, our results indicate that sucrose (or a related disaccharide) contributes to *in vivo* fitness for *S. pneumoniae* in both murine mucosal sites.

ScrH is the major S-6-P hydrolase *in vitro*

ScrH and SusH belong to the glycosyl hydrolase 32 family that includes the invertases (EC #3.2.1.26) (Henrissat and Bairoch, 1993; Pons *et al.*, 1998), which hydrolyse the glycosidic bond in sucrose to yield glucose and fructose. The two proteins share the penta-amino acid sequence (NDPNG), illustrated in Fig. 3A at their N-termini, which is also called the β -fructosidase motif (Ritsema *et al.*, 2004). This pentapeptide motif in turn is part of a large 14-aminoacid motif shown in Fig. 3A that is called the sucrose-binding box (Ritsema *et al.*, 2006) where the aspartate residue is the catalytic nucleophile required for activity (Reddy and Maley, 1990) and is conserved across a large set of invertases from diverse sources (Ritsema *et al.*, 2006) (Fig. 3B). Looking for invertases in different sources, including plants, fungi and

bacteria, reveals that ScrH and SusH cluster into different groups of invertases, with SusH more closely related to invertases from fungi and plants, while ScrH lies within the typical bacterial invertases (Fig. 3B).

To assess the role of the two hydrolases during growth *in vitro*, we tested the ability of the single- and double-hydrolase mutants to grow on sucrose as the sole carbon source in a semi-defined minimal medium (SDMM) (Lacks, 1966). While the *susH* strain grows as well as the wild type (Fig. 3C), the *scrH* strain shows defective growth on sucrose, indicating that ScrH is the dominant sucrose-splitting enzyme *in vitro* (Fig. 3C). However, in the absence of *scrH*, SusH is active as shown by the delayed growth of the *scrH* mutant but no growth of the double-hydrolase null (Fig. 3C). Provision of either *scrH* *in trans* (*susH/scrH/scrH*⁺) or *susH* (data not shown) restores growth of the *susH/scrH* strain on sucrose, confirming the basis of the sucrose-dependent growth phenotype (Fig. 3C). Unexplicably, the *scrH*-complemented strain exhibits a higher growth yield than the wild type and shows delayed normal stationary-phase autolysis.

Based on sequence information and our *in vitro* growth assay results, both genes are likely to be S-6-P hydrolases. To confirm this, and as S-6-P is not commercially available, we assayed the ability of the individual single- and double-hydrolase mutant cell lysates to cleave exogenously added sucrose as reported earlier (Kuramitsu, 1973). This activity was measured by assaying for one of the end-products of the hydrolysis, fructose, using the dinitrosalicylic acid method (Miller, 1959). The *susH* lysate (Fig. 3D) is able to hydrolyse sucrose to the same extent as the wild type consistent with its limited role during *in vitro* growth. In contrast, the *scrH* strain is significantly diminished for sucrose hydrolysis: the remaining residual enzymatic activity in the *scrH* strain is due to SusH as the double-hydrolase null lacks detectable activity. Therefore, ScrH and SusH and their respective linked transporter systems appear to be the only sucrose metabolic systems that are active in *S. pneumoniae* TIGR4.

Regulation of the *scr* and *sus* loci in *S. pneumoniae*

To test the role of the LacI regulators SP1799 (*susR*) and SP1725 (*scrR*) in the regulation of the neighbouring sucrose metabolic genes, we measured the levels of expression of the adjoining genes in the respective regulator mutants compared with wild-type cells grown in rich medium and in SDMM with sucrose as the sole carbon source, using RNase protection assays. The transcripts for the sucrose-specific PTS gene, *scrT*, the sucrose hydrolase, *scrH*, and the putative fructokinase, *scrK*, are derepressed 12-, 4- and 11-fold in the *scrR* strain, respectively, compared with the wild type grown in THY medium (Fig. 4A). Additionally, growth on sucrose induces all three genes 20-, 9- and 30-fold, respectively – levels that are roughly comparable to those in the *scrR* mutant. Thus, ScrR is a repressor of the divergently transcribed sucrose-inducible PTS transporter operon, as illustrated in the model in Fig. 5A.

Analogous results were obtained for the *sus* locus, where loss of *susR* leads to the derepression of the ABC transporter gene, *susT1*, 20-fold and the solute-binding gene, *susX*, 30-fold (Fig. 4B) compared with wild type grown in rich medium. Although not tested, *susT2* is also likely regulated by SusR on the basis of the arrangement of this gene between *susT1* and *susX* in the putative operon. Thus, SusR is a repressor of the adjacent ABC transporter locus (illustrated in Fig. 5A). Although *susH* appears to be the last gene in the *sus* operon, we could not detect its derepression in the *susR* strain suggesting that expression of *susH* might be at low levels *in vitro* or that the *susH* transcript is unstable. Neither repressor (*susR* and *scrR*) regulates the expression of the other operon (*scr* and *sus*) (data not shown).

In contrast to the *scr* locus, sucrose did not induce expression of *susT1*, *susX* nor *susH* in wild-type cells grown in SDMM with sucrose (Fig. 4B). We hypothesized that efficient hydrolysis of the putative inducer S-6-P by ScrH in wild-type cells growing *in vitro* does not allow build-

up of inducer concentration to sufficiently high levels to trigger expression from the *sus* locus. To test this hypothesis, we looked for transcripts of *susX*, *susT1* and *susH* in *scrH* mutant cells that were grown in SDMM with sucrose. Sucrose induces expression of the *susX* and *susT1* genes in the *scrH* mutant (Fig. 4B). This result explains the ability of the *scrH* mutant to grow on sucrose. We infer that lack of the dominant S-6-P hydrolase, ScrH, allows accumulation of high levels of inducer, S-6-P, *in vitro* to allow activation of the *sus* operon (Fig. 5B). It is likely that the *scr* and *sus* loci are 'high' - and 'low' -affinity sucrose-metabolizing systems, respectively, although this remains to be tested. Again, we were unable to detect *susH* transcript, possibly due to unstable or low transcript levels. Nevertheless, we were able to detect SusH sucrose hydrolase activity in the *scrH* mutant when grown in SDMM/sucrose (Fig. 3D), suggesting the gene is expressed.

Discussion

The involvement of carbon metabolic pathways and relevant enzymes in determining *in vivo* fitness and virulence has been demonstrated for several different pathogens (Munoz-Elias and McKinney, 2005; Barelle *et al.*, 2006; Naderer *et al.*, 2006; Tchawa Yimga *et al.*, 2006). The requirement of these pathways during infection has, in a few cases, led to the identification of specific carbon sources that are utilized *in vivo* (Chico-Calero *et al.*, 2002; Chang *et al.*, 2004; Exley *et al.*, 2005). In a previous study, we demonstrated the importance of a LacI family carbon regulator, CcpA, in the fitness of *S. pneumoniae* in the murine lung, bloodstream and nasopharynx (Iyer *et al.*, 2005). Although the mechanistic basis for the *ccpA* attenuation *in vivo* is not clear, the importance of this carbon regulator for colonization and disease established a link between carbon metabolism and *in vivo* fitness and virulence. In this report, we strengthen this link by characterizing two sucrose transport and metabolic systems, *sus* and *scr*, which contribute to *in vivo* fitness. While *scr* is the dominant sucrose uptake and metabolizing system *in vitro*, we show that loss of both *scrH* and *susH*, encoding the respective S-6-P hydrolases for each system, abrogates the ability to utilize sucrose, indicating that both the *sus* and *scr* systems facilitate sucrose uptake and metabolism. In fact, the arrangement of the *scr* operon, its regulation by *scrR* and induction by sucrose are similar to the sucrose-metabolizing locus in the oral pathogen, *Streptococcus mutans* (Sato *et al.*, 1993; Hiratsuka *et al.*, 1998; Wang and Kuramitsu, 2003). Using murine models of carriage and lung infection, we find distinct roles for the two sucrose uptake systems. The *scr* system is largely dispensable for infection of the mouse lung, but plays a role in colonization of the nasopharynx. In contrast, *sus* plays a role in fitness in the mouse lung, but is dispensable in the nasopharynx. The distinct roles of the two sucrose utilization systems in two different host sites reveal niche-specific adaptations that we do not fully understand. While sucrose induces both operons and is transported by *sus* and *scr* *in vitro*, the identity of the carbon sources that are transported into *S. pneumoniae* and that derepress the two operons *in vivo* is unknown. We hypothesize that both systems are involved in the uptake and metabolism *in vivo* of sucrose and/or highly similar disaccharides, and we further suggest that the two operons are differentially induced in the lung and the nasopharynx during infection perhaps in response to different disaccharides. We were also able to complement the *susH* defect in the lung and the *scrH* defect in the nasopharynx. Provision of either gene *in trans* was able to restore growth of the double-hydrolase null on sucrose as the sole carbon source, thus confirming the role of ScrH in sucrose metabolism *in vitro*.

Both the *sus* and *scr* systems are inducible by sucrose and are subject to tight regulation by their cognate LacI family regulators, SusR and ScrR (Fig. 5A). Although S-6-P does not readily induce the *sus* locus in the wild-type strain under the *in vitro* conditions tested, the derepression of the *sus* locus in the *scrH* mutant suggests the need for high intracellular concentrations of S-6-P to counter the repression by SusR (Fig. 5B), a situation that may occur naturally under growth conditions in the lung where *sus* plays a demonstrable role. However, it remains

possible that the natural inducer of the *sus* system *in vivo* is not S-6-P, but is instead a related disaccharide found at relevant concentrations in the mouse lung. Both systems appear to be highly specific for the α -1,2 linkage in sucrose as none of the sucrose isomers tested – palatinose (α -1,6), turanose (α -1,3), maltulose (α -1,4) or leucrose (α -1,5) – could be metabolized by wild type or the *susH/scrH* or *sus/scr* mutants (Table 1).

The entire *scr* locus is conserved in other *S. pneumoniae* serotype strains that have been sequenced, namely serotypes 2 and 19F. While the *sus* system genes, *susR* and *susH*, are present in these other genomes, exact matches for *susT1*, *susT2* and *susX* are not found. While *SusH* is represented within all serotypes (> 97% identity), *SusT1*, *SusT2* and *SusX* have homologues in serotype 3, 14 and 23F and are absent in serotypes 1, 2 and 19F. However, visual inspection of the *susH* and *susR* region in the completely sequenced serotypes, 2 and 19F reveals the existence of a alternative sugar-specific solute-binding/ABC transporter locus in place of *susT1*, *susT2* and *susX*. The sequence dissimilarity in the ABC transporter membrane components and the solute-binding proteins between the serotypes is interesting. Although speculative, it is possible that the requirement of the transporter system *in vivo* may subject the locus to immune pressure and select for sequence divergence among serotypes. Examining sequences upstream of the *sus* operon in all three serotypes revealed a putative *cre* sequence in the intergenic space between the repressor, *susR* and *SusT1* (serotype 4) or the related ABC transporter gene (serotypes 19F and 2). *Cre* sequences are known to be regions that are bound by CcpA, which plays an important role in the catabolite repression or activation of neighbouring genes and contributes to regulation of sugar metabolism and virulence in *S. pneumoniae*. The regulation of the *sus* locus by CcpA and the potential for interplay between CcpA and *SusR* in modulating expression of this locus will be interesting as this may address the mechanistic basis of the previously observed attenuation of the *ccpA* mutant in the lung.

Based on (i) the sucrose-inducible nature of the *sus* locus in the *scrH* mutant, (ii) the presence of the sucrose-binding box (NDPNG), in *SusH*, (iii) the ability of the *scrH* strain to grow on and hydrolyse sucrose and (iv) the loss of this function in the *scrH/susH* null, we infer that the solute-binding protein/ABC-transporter/S-6-P hydrolase trio is an alternative system capable of participating in the uptake and metabolism of sucrose. But, how the *sus* locus is induced *in vivo* is not clear at present. These results establish, for the first time, a link between a specific carbon uptake and metabolic system in *S. pneumoniae* and colonization and disease. Specifically, we show that the metabolism of sucrose and/or a related disaccharide is important during colonization of the nasopharynx and might play a minor role in contributing to survival in the lungs. Amino acids and sugars have recently been shown to be essential carbon sources *in vivo* in both bacterial and protozoan pathogens (Velayudhan *et al.*, 2004; Naderer *et al.*, 2006; Tanzer *et al.*, 2006). In addition, a direct correlation between sucrose and fructose metabolism and *in vivo* fitness has been described in the phytopathogen, *Erwinia amylovora*, and the human dental pathogen, *Streptococcus mutans*, respectively (Bogs and Geider, 2000; Tanzer *et al.*, 2006). The identity of carbon sources that are used by *S. pneumoniae* during infection and colonization, their roles in contributing to fitness and possible virulence gene induction in different host niches are largely unknown. Our observation that two sucrose transport and metabolizing systems contribute to *in vivo* fitness of *S. pneumoniae* in the nasopharynx and to a limited extent, in the lung, represents a significant advance in this direction.

Experimental procedures

Bacterial strains, plasmids and primers for DNA manipulations

Refer to Table 2 for a list of relevant strains and plasmids and the Table S1 for the primers used in this study.

Construction of the SP1799 (*susR*) and SP1795 (*susH*) deletion

The *susR* (SP1799) and the *susH* (SP1795) mutant were constructed by deleting the entire 1001 bp and 1300 bp coding sequence respectively. Approximately 800 bp DNA fragments 5' (F1R1) and 3' (F2R2) of the *susR* and *susH* gene were PCR amplified from AC353 genomic DNA using two primer pair sets, 1799F1–1799R1/1799F2–1799R2 and S6PF2–S6PR2/S6PF1–S6PR1, respectively. Two of the primers in each of the primer pair sets were designed to have a ~20 bp overlap with the 5' and 3' sequences of each of the fragments. The two pieces were then fused together using splicing by overlap extension (SOE) (Horton *et al.*, 1990). The *susR* and the *susH* DNA fragments were then PCR amplified using the primer pair, XM1799–XH1799 and XB95F1–XH95R2, respectively, that add restriction sites at the ends of the product to facilitate cloning into the multiple cloning site of pAC1000, which was then transformed into AC353 cells using standard transformation techniques (Bricker and Camilli, 1999) and mutants were screened for loss of the *susR* and *susH* using PCR and DNA sequencing.

Construction of the *scrR*, *scrH* and *sus* operon deletion

The *scrR* (SP1725), *scrH* (SP1724) and *sus* mutant were constructed by replacing the entire 985 bp, 1450 bp and 6750 bp coding sequence with a CM resistance cassette, *cat*. The *cat* cassette, which has its own promoter and conferred CM resistance on *E. coli* and *S. pneumoniae*, was PCR amplified from pAC1000 (Hava *et al.*, 2003a) by using primers catF1 and NcatR1 (Iyer *et al.*, 2005). Fragments (650–800 bp) containing the regions 5' and 3' of the *scrR*, *scrH* gene were PCR amplified from AC353 genomic DNA by using the primer pair sets, 1725F1–1725R1/1725F2–1725R2, 1724F1–24cat/cat24F2–1724R2 and 1799F1–1799R1/1799F–S6PR2, respectively. Two of the primers in each of the primer sets were designed to have a 20 bp overlap with the 5' and 3' sequences of the *cat* cassette. The *cat* cassette was fused to these two PCR products by SOE as before. The final PCR product was introduced into *S. pneumoniae* TIGR4 by natural transformation as previously described. The double-recombination event was selected for by plating on medium containing 4 μml^{-1} CM and was confirmed by PCR and DNA sequencing.

Construction of the *scr* operon deletion

The *scr* operon deletion was constructed by replacing the entire 5700 bp coding sequence with a spectinomycin (SP) resistance cassette, *aad9*. The *aad9* cassette, which has its own promoter and conferred SP resistance on *E. coli* and *S. pneumoniae*, was PCR amplified from pJR412 (Martin *et al.*, 2000) by using primers spcF1 and spcR1. Approximately 800 bp DNA fragments 5' and 3' of the *sus* operon were PCR amplified from AC353 genomic DNA using the primer pair, 2125F1–2125R1 and 2125F2–1725R2. One primer in each of the primer pairs was designed to have a 20 bp overlap with the 5' and 3' sequences of the *aad9* cassette. The *aad9* cassette was fused to these two PCR products and the final PCR product was introduced into *S. pneumoniae* TIGR4 as described earlier. The double-recombination event was selected for by plating on medium containing 200 $\mu\text{g ml}^{-1}$ SP and was confirmed by PCR and DNA sequencing.

Creation of the double S-6-P hydrolase mutant, *susH/scrH*

The double mutant was created by transforming the *susH* strain with genomic DNA from the *scrH* mutant, using natural transformation as previously described. The recombination event was selected for by plating on medium containing 4 $\mu\text{g ml}^{-1}$ CM and was confirmed by PCR and DNA sequencing.

Construction of the *susH* and *scrH* complementation strains

The *susH* and *scrH* complementation strategy involved making transcriptional fusions of *susH* and *scrH* with the SP resistance gene, *aad9*, so that the genes are driven by the *aad9* promoter and the construct was recombined into the LacE (SP0474-SP0478) locus (Iyer *et al.*, 2005) to create strains, RI2587 and RI2603. For complementation of *scrH* *in vitro*, we also made another construct, where *scrH* was similarly transcriptionally fused to *aad9* (as above) but was placed downstream from its putative regulatory upstream region in an attempt to preserve wild type-like regulation of *scrH* to create RI2650. This we also refer to as Native *scrH* (*NscrH*). To complement *scrH* in the nasopharynx, we additionally placed a transcriptional terminator at the end of the *aad9* gene in the *NscrH* construct to allow for more controlled expression *in vivo*, to create strain RI2678. For complementation of the *susH* mutation, *susH*, along with its native ribosome binding site, was amplified by PCR from AC353 genomic DNA with primers susHF-susHR. This PCR fragment was used as the template for another PCR amplification step using the primers spsusH and susHR. This PCR adds a 33 bp sequence at the 5' end that is homologous to the 3' end of the *aad9* (SP resistance cassette) gene. The primers suslac and NLacER were used to PCR amplify the 3' end of the LacE locus (LacE dn) from AC353 genomic DNA. The suslac primer has a 20 bp sequence at the 5' end that is homologous to the 3' end of the *susH* gene. The suslac-NLacER PCR fragment was joined to the spsusH-susHR fragment using SOE, to create the susH-LacE dn piece. The primers NLacEF and spsusH were used to amplify the region 5' of the LacE locus (LacE up) along with the *aad9* gene from the *ccpA*⁺ strain, RI1933, as described previously. The LacE up-*aad9* piece was then joined by SOE to the susH-LacE dn fragment to create the final LacE up-*aad9*-*susH*-LacE dn construct that was introduced into the *susH/scrH* strain to create RI2587. The double-recombination event was selected for by plating on medium containing 200 μml^{-1} SP and was confirmed by PCR and DNA sequencing.

For complementation of the *scrH* mutation, *scrH* including its native ribosome binding site, was amplified by PCR from AC353 genomic DNA using primers, scrHF-scrHR. This PCR fragment was used as the template for another PCR amplification step using the primers spscrH and scrHR. This PCR adds a 33 bp sequence at the 5' end that is homologous to the 3' end of the *aad9* gene. The primers sclac and NLacER were used to PCR amplify the 3' end of the LacE locus (LacE dn) from AC353 genomic DNA. The sclac primer has a 20 bp sequence at the 5' end that is homologous to the 3' end of *scrH*. The sclac-NLacER PCR fragment was joined to the spscrH-scrHR fragment using SOE, to create the *scrH*-LacE dn piece. The primers NLacEF and spcR1 were used to amplify the region 5' of the LacE locus (LacE up) along with the *aad9* gene from the *ccpA*⁺ strain, RI1933. The LacE up-*aad9* fragment was then joined by SOE to the *scrH*-LacE dn piece to create the final LacE up-*aad9*-*scrH*-LacE dn construct that was introduced into the *susH/scrH* strain to create RI2603. The double-recombination event was selected for by plating on medium containing 200 μml^{-1} SP and was confirmed by PCR and DNA sequencing.

In another strategy for complementation of the *scrH* mutation, *scrH*, along with a putative regulatory region 360 bp upstream including its native ribosome binding site, was amplified by PCR from AC353 genomic DNA. Primers scrupF-scrupR amplified the 360 bp putative regulatory region upstream of *scrH* to create *scrH* (upstream). Separately, primers scrHF-scrHR were used to amplify the entire ~1800 bp *scrH* gene. Using this PCR product as the template, primers upscrHF-scrHR was used to re-amplify *scrH*, so that primer upscrHF creates a 22 bp addition at the 5' end of *scrH* facilitating fusion of this piece with the scrupF-scrupR product using SOE to create *scrH* (upstream)-*scrH*. The primers sclac and NLacER were used to PCR amplify the 3' end of the LacE locus (LacE dn) from AC353 genomic DNA. The sclac primer has a 25 bp sequence at the 5' end that is homologous to the 3' end of *scrH*. *scrH* was then fused to the sclac-NLacER fragment to create *scrH*-LacE dn. Subsequently, the *scrH*

(upstream)-*scrH* fragment was fused to the *scrH*-LacE dn fragment by SOE to create *scrH* (upstream)-*scrH*-LacE dn. In a separate reaction, primers NLacEF and upspcR1 were used to amplify the LacE up-*aad9* region from RI1933 (Iyer *et al.*, 2005). The primer upspcR1 creates a 29 bp sequence at the 3' end of the LacE up-*aad9* fragment that is homologous to the 5' end of *scrH* (upstream)-*scrH*-LacE dn. The NLacEF-upspcR1 fragment was then fused to the *scrH* (upstream)-*scrH*-LacE dn piece by SOE to create the final ~4200 bp construct, LacE up-*aad9*-*scrH* (upstream)-*scrH*-LacE dn that was introduced into the *susH/scrH* strain to create RI2650. The double-recombination event was selected for by plating on medium containing 200 $\mu\text{g ml}^{-1}$ SP and was confirmed by PCR and DNA sequencing.

In order to complement the *susH/scrH* defect in nasopharyngeal colonization, we placed a transcriptional terminator at the end of the *aad9* gene in the *NscrH* construct. We chose the genomic transcriptional terminator downstream from the SP0479 gene (genomic co-ordinates 459144–459094) for this purpose. We PCR amplified the *lacE* (up)-*aad9* region from RI1933 genomic DNA using primers NLacEF and TTR to create LacEF (up)-TTR. The TTR primer has a 20 bp homology with the 3' end of the *aad9* gene and additionally has a 23 bp overhang that is part of the terminator. Separately, we used primers TTF and scrHR to PCR amplify the *scrH* (upstream)-*scrH* fragment from RI2650 genomic DNA to create TTF-*scrH* (upstream)-*scrH*. The TTF primer has a 29 bp region of homology with the 5' end of the *scrH* (upstream) fragment. The TTF primer also has a 21 bp overhang at the 5' end that is part of the terminator. We PCR amplified the TTF-*scrH* (upstream)-*scrH* fragment using primers TTF1 and scrHR to create TTF1-*scrHR*. The TTF1 primer shares at its 3' end 20 bp homology with the 5' end of TTF and in addition 20 bp with the 5' end of TTR. This step creates the transcriptional terminator (tt) in the fragment. We then joined the LacEF (up)-TTR fragment to TTF1-*scrHR* to create LacEF (up)-*aad9*-tt-*scrH* (upstream)-*scrH* using SOE. As described earlier, we fused LacEF (up)-*aad9*-tt-*scrH* (upstream)-*scrH* to LacE (dn) using SOE to create the final ~4.3 kb LacEF (up)-*aad9*-tt-*scrH* (upstream)-*scrH*-LacE (dn), which was introduced into the *susH/scrH* strain to create RI2678. The double-recombination event was selected for by plating on medium containing 200 $\mu\text{g ml}^{-1}$ SP and was confirmed by PCR and DNA sequencing.

Bacterial growth and media

Streptococcus pneumoniae was routinely grown in Todd–Hewitt broth with 1% yeast extract (THY) or on blood agar plates containing 100 μml^{-1} streptomycin (SM) at 37°C in a 5% CO₂ atmosphere. For broth cultures, 5 $\mu\text{l ml}^{-1}$ Oxyrase (Oxyrase) was added in addition and cultures were typically grown to a final optical density at 600 nm (OD₆₀₀) of 0.5–0.6. To test growth on sucrose as the sole carbon source and sucrose hydrolysis assays, *S. pneumoniae* was grown in SDMM without BSA, containing appropriate antibiotics (SM: 100 $\mu\text{g ml}^{-1}$; CM: 4 $\mu\text{g ml}^{-1}$; SP: 200 $\mu\text{g ml}^{-1}$). The concentration of sugar was 10 mM in all cases. There was negligible growth in SDMM in the absence of added sugar.

Animal infections

All animal infections were performed with 8- to 12-week-old male/female Swiss–Webster mice (Taconic Laboratories). Mice were provided with continuous food and water. Bacteria were recovered from plates of tryptic soy agar (TSA) with 5% sheep's blood, subcultured in THY and grown at 37°C for 4 h to an OD₆₀₀ ~0.4–0.5. Lung and nasopharyngeal infections in mice were performed as described earlier (Hava and Camilli, 2002; Iyer *et al.*, 2005). Lung infection was carried out for 42 h and nasopharyngeal colonization for 6–7 days, after which the mice were sacrificed and counted for bacteria as described before. Housing of mice and all procedures were performed according to procedures approved by the Tufts University Institution of Animal Care and Use Committee.

RNase protection assays

Total RNA was isolated from 10 ml of *S. pneumoniae* wild-type, *susR* and *scrR* mutant cells grown in THY supplemented with SM (100 $\mu\text{g ml}^{-1}$) and Oxyrase (5 $\mu\text{l ml}^{-1}$) or for sucrose induction, from wild-type cells grown in SDMM containing 10 mM sucrose, 30 units/ml catalase and SM (100 $\mu\text{g ml}^{-1}$). RNA was isolated with a Qiagen RNeasy kit as described earlier. In the case of *scrH* mutants, cells were grown up in THY as earlier to $\text{OD}_{600} \sim 0.4-0.5$. The cells were centrifuged at 4000 r.p.m. for 10 min and the pellet was re-suspended in 10 ml of SDMM containing 10 mM sucrose for 2 h at 37°C and 5% CO_2 prior to RNA isolation. Template DNA for the generation of riboprobes was PCR amplified with primer sets, NplyF-rpapy, plyF-rpapy, rp1795-1795R0, rp1796-1796R, rp1798-1798F, rp1724-1725F0, rp1722-1722R and rp1721-1721F.

Sucrose hydrolysis assays

The hydrolysis of sucrose by *S. pneumoniae* wild-type and mutant lysates was monitored over time by measuring the accumulation of the end-product, fructose, using the dinitro-salicylic acid-phenol (DNS) colorimetric assay as described. Wild-type, *susH*, *scrH* and *susH/scrH* cells were grown up in duplicate in 5 ml of THY (1% yeast extract) to an $\text{OD}_{600} \sim 0.4-0.5$. The entire suspension was centrifuged at 4000 r.p.m. for 5 min to pellet the cells. The pellets were washed once in 1 ml of pre-warmed sugar-less SDMM and re-suspended in 1 ml of SDMM. After centrifugation at 4000 r.p.m. for 5 min, the washed pellets were re-suspended in 1 ml of SDMM and inoculated into 10 ml of pre-warmed SDMM with or without 10 mM sucrose and incubated at 37°C and 5% CO_2 for 120 min to allow induction of *susH* or *scrH* genes. At various time points during the incubation (at ~30–40 min intervals), 1 ml of aliquots were drawn out and centrifuged at 10000 r.p.m. for 1 min and washed once with 500 μl of warm 100 mM K_2HPO_4 buffer (pH 7.0). After centrifugation, the pellets were re-suspended in 400 μl of warm 100 mM K_2HPO_4 buffer (pH 7.0). Five microlitres of 10% Triton X-100 was added to the suspension to get a final detergent concentration of 0.1% followed by the addition of 100 μl of 1M sucrose in 100 mM K_2HPO_4 buffer (pH 7.0) to get a final sucrose concentration of 200 mM. The suspension was mixed by inverting and incubated at 37°C for 70 min. In parallel, we also prepared a similar blank reaction that does not have cells. At the end of the incubation, 300 μl from each reaction was withdrawn and added to a tube containing 300 μl of DNS (Miller, 1959), and incubated at 90°C for 15 min until the development of a red brown colour. The tubes were then transferred to an ice bucket and 100 μl of 40% Rochelle's salt was added to each reaction and the tubes were left of ice for ~5 min to cool the contents. The absorbance of the reaction was measured at 575 nm against the blank, and the amount of fructose at the end of the reaction was calculated by reading from a separately constructed fructose standard curve.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to members of the Camilli lab for helpful discussions and suggestions during the course of this work. A.C. is an investigator of the Howard Hughes Medical Institute (HHMI). This work was supported by the HHMI.

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Fig. 1.

The S-6-P hydrolase, *susH*, and the *sus* operon contribute to survival of *S. pneumoniae* in the murine lung. Loss of the S-6-P hydrolase, *susH*, or loss of the *sus* operon, *sus*, causes attenuation in the pneumonia model of infection in competition with wild-type cells. Loss of the second S-6-P hydrolase, *scrH*, causes only mild attenuation, with no additive attenuation in the double S-6-P hydrolase null, *susH/scrH*, or the double operon mutant, *sus/scr*, in competitions with wild type. Provision of a single copy of the *susH* gene *in trans* in the double S-6-P hydrolase null complements the *in vivo* defect.

Fig. 2.

The S-6-P hydrolase, *scrH*, contributes to colonization of the murine nasopharynx. Loss of the S-6-P hydrolase, *scrH*, causes attenuation in the nasopharyngeal colonization model of infection. In contrast, although the *susH* mutant is not attenuated, the double S-6-P hydrolase mutant *susH/scrH* is significantly more attenuated than *scrH* ($P = 0.02$) indicating that SusH might be active in the *scrH* mutant. Provision of *scrH* *in trans* completely rescues the colonization defect in the *susH/scrH* mutant.

Fig. 3.

The S-6-P hydrolase, *scrH*, is required for growth on sucrose *in vitro*.

A. Alignment of the conserved sucrose-binding box sequences of SusH and ScrH along with those of some representative invertases from Gram-positive and Gram-negative bacteria, fungi and plants. The conserved β -fructosidase motif is underlined and the conserved aspartate residue is in bold.

B. Phylogenetic tree describing the clustering of SusH and ScrH in relation to S-6-P hydrolases (invertases) from diverse bacterial, fungal and plant sources. SusH is more related to the fungal and plant enzymes while ScrH clusters with typical bacterial invertases. All the members share the sucrose-binding box motif.

C. *scrH* has a pronounced growth defect on sucrose while *susH* is dispensable. Loss of both *scrH* and *susH* (*susH/scrH*) abrogates the ability of *S. pneumoniae* to utilize sucrose as the sole carbon source *in vitro*. Provision of *scrH* *in trans* restores growth of the *susH/scrH* mutant on sucrose.

D. Wild-type and *susH* lysates of *S. pneumoniae* grown on sucrose show 'sucrolytic' activity with comparable kinetics of fructose accumulation over time. The *scrH* lysate shows severely decreased rate of sucrose hydrolysis. There is no detectable sucrose hydrolysis activity in the S-6-P hydrolase double mutant, *susH/scrH*.

Fig. 4.

Ribonuclease protection assays (RPAs) depicting regulation of the *scr* and *sus* operons by ScrR and SusR and sucrose. RNA was isolated from wild type, *scrR*, *susR* or *scrH* mutant *S. pneumoniae* strains after growth in rich medium (THY) or SDMM with sucrose as the sole carbon source (Sucrose). Transcripts were detected in 1–3 µg of total RNA by RPA. Band intensities were quantified and normalized to the intensity of the pneumolysin gene (*ply*) band. Radioactivity per lane per probe was arbitrarily fixed at 90 000 cpm.

A. ScrR is a repressor of *scrH*, *scrT* and *scrK* (*scrR*) and growth on sucrose induces *scrH*, *scrT* and *scrK* (sucrose) in wild-type cells. Average intensity relative to the wild type from two independent experiments is reported above each lane. U indicates undigested *ply* (lane 1), *scrH* (lane 2), *scrT* (lane 3) and *scrK* (lane 4) probes.

B. SusR is a repressor of *susT1* and *susX* (*susR*) but growth on sucrose does not induce expression of *susT1*, *susX* or *susH* in wild-type cells. Growth on sucrose induces expression of *susT1* and *susX* in the *scrH* mutant (*scrH*). Average intensity relative to the wild type from two independent experiments is reported above each lane, where applicable. Left: U indicates undigested *susH* (lane 1), *susT1* (lane 2 – upper band) and *susX* (lane 2 – lower band) probes.

Right: U indicates undigested *susT1* (lane 1 – upper band), *ply* (lane 1 – middle band), *susX* (lane 1 – lower band) and *susH* (lane 2) probes.

Fig. 5.

A model describing the hierarchical induction of the *sus* and *scr* systems by the putative inducer sucrose-6-phosphate (S-6-P).

A. *susR* and *scrR* genes code for the repressors, SusR and ScrR, which normally repress the expression of the downstream genes in the *sus* and *scr* operons respectively. S-6-P is formed inside the cell concomitant with entry via ScrT, the sucrose PTS transporter. Once inside, S-6-P derepresses the *scr* system leading to the expression of the S-6-P hydrolase, ScrH and the fructokinase, ScrK. ScrH activity is the predominant 'sucrolytic' activity in wild-type cells under these conditions. ScrH hydrolyses S-6-P to glucose-6-phosphate (G-6-P) and fructose. A small fraction of sucrose could enter the cell via the ABC transporter, SusT1/SusT2 and is converted to S-6-P via the action of an unknown kinase.

B. In the *scrH* mutant, influx of S-6-P via ScrT causes derepression of the *scr* operon leading to expression of *scrT* and *scrK*. However, the lack of *scrH* leads to an increase in intracellular concentrations of S-6-P (wide, filled, upward-directed arrow). This condition triggers derepression of the *sus* operon causing the expression of the ABC system, *susT1/susT2*, the solute-binding component, *susX*, and the second S-6-P hydrolase, *susH*. The *sus* pathway hydrolase, SusH, hydrolyses S-6-P to G-6-P and fructose.

Table 1

Growth of the S-6-P hydrolase mutant (*susH/scrH*) and the double operon null (*sus/scr*) on glucose, fructose, sucrose and sucrose isomers.^a

Strain	Glucose	Fructose	Sucrose	Palatinose	Turanose	Maltulose	Leucrose
Wild type	+	+	+	-	-	-	-
<i>sus/scr</i>	+	+	-	-	-	-	-
<i>susH/scrH</i>	+	+	-	-	-	-	-

^a Individual sugar (10 mM) in SDMM.

Table 2

Relevant strains and plasmids.

Strain/plasmid	Relevant genotype or phenotype	Source or reference
<i>S. pneumoniae</i>		
AC353	Spontaneous Sm ^r derivative of TIGR4	
RI2455	AC353 <i>scrH::cat</i>	This work
RI2436	AC353 <i>susH</i>	This work
RI2456	RI2436 <i>scrH::cat</i>	This work
RI2480	AC353 <i>scr::aad9</i>	This work
RI2479	AC353 <i>sus::cat</i>	This work
RI2481	RI2479 <i>scr::aad9</i>	This work
RI2587	RI2456 (SP0474-SP0478:: <i>aad9-susH</i>)	This work
RI2603	RI2456 (SP0474-SP0478:: <i>aad9-scrH</i>)	This work
RI2650	RI2456 (SP0474-SP0478:: <i>aad9-NscrH</i>)	This work
RI1932	AC353 <i>ccpA::cat</i>	Iyer <i>et al.</i> (2005)
RI1933	RI1932 (SP0474-SP0478:: <i>aad9-ccpA</i>)	Iyer <i>et al.</i> (2005)
RI2678	RI2456 (SP0474-SP0478:: <i>aad9</i> (tt)- <i>NscrH</i>)	This work
<i>E. coli</i>		
AC578	DH5a λ pir(pR412); Ap ^r , Sp ^r	Iyer <i>et al.</i> (2005)
AC1000	DH5a λ pir(pAC1000); Ap ^r , Cm ^r	Iyer <i>et al.</i> (2005)
Plasmids		
pAC1000	<i>S. pneumoniae</i> suicide vector; Cm ^r	Hava <i>et al.</i> (2003a)
pR412	Has <i>magellan5</i> minitransposon; Ap ^r , Sp ^r	Martin <i>et al.</i> (2000)