

## Peptide Permeases from *Streptococcus pneumoniae* Affect Adherence to Eucaryotic Cells

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To gain access to tissues within the human host, *Streptococcus pneumoniae* initially colonizes the nasopharynx and then interacts with glycoconjugates on the surfaces of target cells at various sites of infection. Although pneumococcal adhesins are currently unknown, exported proteins on the bacterial surface are potential candidates. To identify bacterial elements involved in this process, mutants of *S. pneumoniae* with defects in exported proteins were screened for the inability to adhere to cells representative of three *in vivo* niches: (i) agglutination of bovine erythrocytes, which reflects adherence to cells which reside in the nasopharynx; (ii) human type II pneumocytes (lung cells [LC]), representing the alveolar site of infection; and (iii) human vascular endothelial cells (EC), representing the endovascular site. The capacity of the mutants to adhere during the course of pneumococcal disease was also assessed by using cytokine-activated LC and EC. All of the 30 mutants analyzed produced hemagglutination values comparable with those of the parent strain. Four independent mutants demonstrated a greater than 50% decrease in adherence to both LC and EC. Sequence analysis of the altered alleles from these strains showed that mutations had occurred in two previously identified loci, *plpA* and *ami*, which belong to the family of genes encoding protein-dependent peptide permeases. Mutations in the *ami* locus resulted in an inability to recognize the GalNAc $\beta$ 1-4Gal glycoconjugate receptor present on resting LC and EC, whereas mutations in *plpA* resulted in a failure to recognize a GalNAc $\beta$ 1-3Gal glycoconjugate receptor also present on resting cells. Mutations in neither allele affected recognition of GlcNAc receptors present on cytokine-activated LC and EC. These results suggest that peptide permeases modulate pneumococcal adherence to epithelial and endothelial cells either by acting directly as adhesins or by modulating the expression of adhesins on the pneumococcal surface during the initial stages of colonization of the lung or the vascular endothelium.

*Streptococcus pneumoniae* is a gram-positive pathogen that adapts to several different niches within the eucaryotic host, causing a variety of diseases (for a recent review, see reference 42). As for most bacteria, the dynamic interaction between pneumococci and resident host cells at the site of infection is the basis for bacterial survival and the development and progression of disease. Therefore, identification of the molecular elements that participate in this association is essential for describing pneumococcal disease.

A common resident of the nasopharynx, pneumococci can be cultured from 40 to 60% of the healthy nasal flora of the general population at any given time (9). Several studies suggest pneumococcal binding to glycoconjugate receptors present on the surfaces of resident epithelial cells (EC) (5, 6). With infection, pneumococci will invade the alveoli of the lower respiratory tract to cause lobar pneumonia. Early stages of disease are characterized by the appearance of bacteria closely lining the alveolar epithelium (28, 43). *In vitro* studies from our laboratory suggest preferential attachment to type II pneumocytes (lung cells [LC]) (13). Pneumococci can also invade the bloodstream by crossing the alveolar capillaries, leading to bacteremia, and in certain instances will cross the vascular cell layer of the blood-brain barrier to cause meningitis. Therefore, attachment and migration across EC barriers is a critical aspect for the progression of systemic infection.

Bacterial adherence to host cells commonly involves the recognition of eucaryotic surface receptors that contain glyco-

conjugates. Pneumococcal adherence in the nasopharynx is presumably mediated by *N*-acetyl-D-glucosamine  $\beta$ 1-3 galactose (GlcNAc $\beta$ 1-3Gal) glycoconjugate receptors (5). Work from our laboratory suggests that adherence to both human LC and vascular EC involves three classes of receptors. On resting host cells, pneumococci bind to *N*-acetyl-D-galactosamine  $\beta$ 1-3 galactose (GalNAc $\beta$ 1-3Gal)- or *N*-acetyl-D-galactosamine  $\beta$ 1-4 galactose (GalNAc $\beta$ 1-4Gal)-containing receptors (13). We have also shown that vascular EC and LC respond to host inflammatory stimuli with the induction of a third class of glycoconjugate receptor which contains a GlcNAc specificity (11, 14). Human vascular EC express this new receptor following preincubation with the proinflammatory cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1) and the chemokine thrombin (11). In contrast, induction of LC occurs following exposure to IL-1. This new receptor promotes a 70% increase in pneumococcal adherence to TNF- and thrombin-stimulated EC and a 30% increase in adherence to both EC and LC stimulated with IL-1.

For many pathogenic bacteria, exported proteins are determinants of virulence that participate in the process of adherence to eucaryotic cells. To determine a molecular basis for the interaction between pneumococci and target host cells from the perspective of the bacteria, we developed a genetic strategy that generates mutants with defects in exported proteins (26). These pneumococcal strains were then screened for loss of function in assays that represent adherence to (i) target cells of the nasopharynx, (ii) cultured LC, and (iii) vascular EC.

### MATERIALS AND METHODS

**Strains and media.** The pneumococcal strains used in this study and their relevant characteristics are summarized in Table 1. Libraries of pneumococcal

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TABLE 1. *S. pneumoniae* strains used in this study

Strain	Relevant characteristic(s)	Integrated plasmid	Reference or source
R6x	<i>hex</i> , parent strain	None	41
SPRU16	<i>spxB-phoA</i> fusion	<i>spxB</i> ::pHRM104	37
SPRU30	<i>glpD-phoA</i> fusion	<i>glpD</i> ::pHRM100	25
SPRU58b	<i>plpA-phoA</i> fusion	<i>plpA</i> ::pHRM104	25
SPRU98	<i>plpA-phoA</i> fusion	<i>plpA</i> ::pHRM104	25
SPRU121	<i>amiA-phoA</i> fusion	<i>amiA</i> ::pHRM104	25
SPRU148	<i>amiC-phoA</i> fusion	<i>amiC</i> ::pHRM104	25
LM34	<i>pspA</i>	<i>pspA</i> ::pVA891	Larry S. McDaniel

mutants containing random insertions of the shuttle vector pHRM100 or pHRM104 were from a previously described work (26). *S. pneumoniae* was routinely grown on plates of tryptic soy agar supplemented with 3% sheep blood and in a semisynthetic liquid medium as described elsewhere (25, 26).

**Mutagenesis and pneumococcal transformation.** Allelic disruption in pneumococci was achieved by insertional inactivation with the pneumococcus-*Escherichia coli* shuttle vectors pJDC9, pHRM100, and pHRM104 (10, 26). The vectors pHRM100 and pHRM104 were used to create translational fusions to a truncated version of the gene for a bacterial alkaline phosphatase (*phoA*), while pJDC9 was used for targeted mutagenesis without a productive gene fusion. The details for using these vectors and the transformation of homologous DNA into pneumococci have been previously described (10, 25, 26). The nature of the insertions in most of the mutants was confirmed by PCR with chromosomal DNA from each of the mutants and oligonucleotides directed toward the vector and flanking DNA. These results were described previously (25, 26).

**Recombinant DNA techniques.** Techniques for manipulating DNA, including plasmid preparations, restriction endonuclease digestions, sequencing, ligations, transformation into *E. coli*, and gel electrophoresis, were performed according to standard protocols (34).

**Hemagglutination of neuraminidase-treated bovine erythrocytes.** Neuraminidase-treated bovine erythrocytes which had exposed GlcNAc $\beta$ 1-3Gal glycoconjugates were prepared as described previously (5). Bovine erythrocytes were suspended in 10 ml of Dulbecco's phosphate-buffered saline (DPBS) and incubated with an equal volume of neuraminidase (1 U/ml) for 30 min at 37°C, washed twice by centrifugation (for 3 min at 13,000  $\times$  g), resuspended in albumin-containing DPBS, and diluted 100-fold from a 5% (vol/vol) stock. Hemagglutination was assessed by light microscopy following incubation of equal volumes (10  $\mu$ l) of erythrocytes with pneumococci (10<sup>9</sup> CFU/ml) for 5 min at room temperature. Hemagglutination was visually scored in a range from 1+ (<5% of the erythrocytes aggregated, considered negative) to 4+ (instant aggregation). Each strain was tested on at least six separate occasions.

**Culture of LC and vascular EC.** The LC line A549 (American Type Culture Collection) was cultured in the nutrient mixture Ham F12 medium supplemented with 10% fetal calf serum, while primary cultures of human umbilical vein EC (passage 1; Clonetics Corp., San Diego, Calif.) were grown in medium 199 as previously described (13, 17). At confluence, the cells were prepared for subculture with trypsin-0.05% EDTA. For adherence assays, cells were transferred to 60-well Terasaki culture dishes coated with fibronectin (50  $\mu$ g/ml) and cultured for another 24 to 48 h to form confluent monolayers, which were subsequently washed twice with medium 199 before incubation with bacteria.

**FITC labeling of pneumococci.** Pneumococci were harvested from tryptic soy agar-3% sheep blood plates, resuspended in 1 ml of DPBS, and incubated with fluorescein isothiocyanate (FITC; 1 mg/ml) as previously described (13, 18). The bacteria were washed twice by centrifugation (for 3 min at 13,000  $\times$  g), resuspended in 1 ml of an albumin-containing buffer, and diluted to concentrations that ranged between 10<sup>5</sup> and 10<sup>7</sup> CFU/ml. Labeling with FITC had no significant effect on viable counts.

**Adherence of FITC-labeled pneumococci to resting and activated eucaryotic cells.** Adherence of pneumococci to LC and EC was assessed as previously described (13, 18). For adherence to cells activated by proinflammatory molecules, monolayers of LC were preincubated with 5 ng of IL-1 per ml for 4 h at 37°C and EC were preincubated with 5 ng of TNF per ml for 3 h at 37°C (11). Prior to the adherence assay, the monolayers were washed twice in medium 199. FITC-labeled bacteria (10<sup>5</sup> to 10<sup>7</sup> CFU/ml, 10  $\mu$ l per well) were incubated with either resting or activated EC or LC monolayers for 30 min at 37°C. For experiments that define the ability of sugars to inhibit adherence, FITC-labeled pneumococci (2  $\times$  10<sup>7</sup> CFU/ml) were preincubated for 15 min at room temperature with the monosaccharide GlcNAc at a final concentration of 50 mM, the glycoconjugate asialo-GM2, which has the terminal sugar GalNAc $\beta$ 1-4Gal, or globoside, which has the terminal sugar GalNAc $\beta$ 1-3Gal, at a final concentration of 0.1 mM, or an albumin-containing buffer as a control (11, 13). It has previously been determined that these sugars best represent glycoconjugate receptor populations for pneumococci on resting (asialo-GM2 and globoside) and activated (GlcNAc) human vascular EC and LC (11, 13). Bacteria were centrifuged (for 3 min at 13,000  $\times$  g) to remove excess sugar, resuspended to a concentration of 10<sup>7</sup>

CFU/ml in albumin-containing DPBS, and added to the eucaryotic cells. Non-adherent bacteria were removed by washing the monolayers three to five times with medium 199. All monolayers were then fixed in 2.5% glutaraldehyde. Adherent bacteria were counted visually with an inverted microscope (Diaphot-TMD; Nikon Inc., Melville, N.Y.) equipped for fluorescence with an IF DM-510 filter. Adherence is expressed as the number of attached bacteria per 100 eucaryotic cells counted in a 40 $\times$  field (13, 18). Multiple values were averaged, and each experiment was performed on at least six separate occasions. Inter- and intra-assay coefficients of variation for the adherence assay were 18 and 15%, respectively.

**Adherence of pneumococci to immobilized sugars.** Pneumococci were tested for adherence to a number of sugars which have been identified as effective analogs of glycoconjugate-containing receptors present on eucaryotic cells. Solid-phase binding assays were carried out as previously described (13). The monosaccharide GlcNAc and two complex glycoconjugates (asialo-GM2 [GalNAc $\beta$ 1-4Gal] and globoside [GalNAc $\beta$ 1-3Gal]) were tested. Stock solutions of the monosaccharide (100 mM) and the glycoconjugates (2 mM) were prepared in 10% (vol/vol) chloroform in methanol. Solutions of monosaccharide (50 mM) and the glycoconjugates (0.1 mM) were made in methanol. Methanol alone served as a control. Terasaki 60-well culture dishes were coated with 10  $\mu$ l per well and allowed to evaporate to dryness at 4°C. The wells of the microtiter plate were then blocked by incubation (overnight, 37°C) with 5% (wt/vol) bovine serum albumin (BSA) in DPBS. The excess BSA-containing buffer was decanted, and the wells were washed once with 0.05% BSA in DPBS. Fluorescein-labeled pneumococci (10<sup>7</sup> CFU/ml) were allowed to adhere to the plates for 30 min at 37°C, and adherence was quantified visually with an inverted fluorescence microscope. Glycoconjugates were purchased from Sigma (St. Louis, Mo.).

**Statistics.** Differences between groups of adherence assays were evaluated by the Wilcoxon signed ranks test. All results are expressed as means and standard deviations from at least six experiments.

## RESULTS

**Generation of pneumococcal mutants with defects in exported proteins.** To alter the surface of the pneumococcus, insertion duplication mutagenesis coupled to translational *phoA* (alkaline phosphatase) fusions was used to identify and generate a bank of mutants with defects in exported proteins (26). *PhoA*<sup>+</sup> mutants were then assessed in three different in vitro assays that measure adherence to eucaryotic cells.

**Identification of pneumococci deficient in adhesion to LC and EC.** Work from our laboratory has previously described an in vitro adherence assay based on the binding of fluorescence-labeled pneumococci to both LC and EC (13). The binding of pneumococci was dose dependent and not affected by the presence or absence of capsules from a variety of serotypes. A bank of 30 *PhoA*<sup>+</sup> mutants was screened for a decrease in adherence to both resting LC and EC. We identified three mutants, SPRU58b, SPRU98, and SPRU121, which consistently adhered 50 to 60% less avidly than the R6x parent or a strain with a deletion in *PspA*, which is another pneumococcal surface protein (44, 45) (Fig. 1B). Identical results were obtained with adherence to EC (data not shown). The mutations associated with each allele were genetically transferred to the parent strain by targeted mutagenesis with a vector (pJCD9) that altered the alleles but did not synthesize a productive *PhoA* fusion. These strains were also adhesion deficient to the same degree as their ancestors, which shows that a mutation in each allele was responsible for the loss of function and that the expression of a *PhoA* fusion protein did not contribute to the phenotype. At an input concentration of 10<sup>7</sup> CFU/ml, levels of adherence to EC of the *PhoA*<sup>+</sup> and *PhoA*<sup>-</sup> mutants created with the allele recovered from SPRU121 were 106  $\pm$  17 and 118  $\pm$  10, respectively, bacteria per 100 eucaryotic cells, and levels of adherence to LC were 103  $\pm$  10 and 123  $\pm$  9, respectively, bacteria per 100 eucaryotic cells. A value of 290  $\pm$  12 adherent bacteria per 100 eucaryotic cells was obtained with the parent strain. Similar decreases in adherence were observed for corresponding strains created with the alleles recovered from SPRU98 and SPRU58b (data not shown).

**Pneumococcus-induced hemagglutination mediated by GlcNAc $\beta$ 1-3Gal glycoconjugates.** It has previously been shown

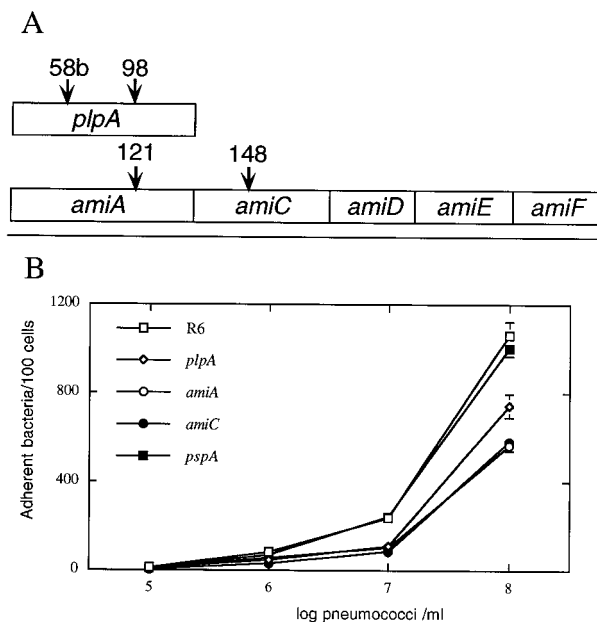


FIG. 1. Adherence of pneumococcal mutants to cultured cells. (A) Genetic maps of the recovered loci of adhesion-deficient mutants of *S. pneumoniae*. (B) Adherence of pneumococcal mutants with defects in *amiA*, *amiC*, *plpA*, and *pspA* to cultured human vascular EC. Adherent bacteria were determined as the number of attached bacteria per 100 cells. Results are presented as the means  $\pm$  standard deviations for duplicate wells in at least six independent experiments. Similar results were obtained for cultured LC (data not shown).

that neuraminidase treatment of bovine erythrocytes exposes GlcNAc $\beta$ 1-3Gal glycoconjugates which represent the proposed nasopharyngeal cell receptor (5). Therefore, we developed a bacterium-induced hemagglutination assay to test these mutants for loss of function. In six separate experiments, at an input concentration of  $10^9$  CFU/ml, SPRU58b, SPRU98, and SPRU121 gave agglutination values of between 2+ and 3+, which are comparable to values obtained with the parent strain (data not shown). Another PhoA<sup>+</sup> strain, SPRU16 (37), was hemagglutination negative (1+) and served as a control. This finding shows that these mutants had not lost the ability to bind the putative glycoconjugate receptor for target cells of the nasopharynx.

**Molecular analysis of the loci associated with the adherence-deficient mutants.** Molecular analysis of the altered loci from the three adhesion-deficient strains showed that gene disruption had occurred in two previously identified alleles, *plpA* (25) and *amiA* (3, 4). Sequence analysis revealed that vector insertion in SPRU58b and SPRU98 had occurred in two separate sites for *plpA* (26), while mutant SPRU121 contained an insertion in *amiA* (Fig. 1A). These loci encode members of the family of bacterial permeases, which usually consist of an exported, lipid-anchored, substrate-binding protein and a complex of membrane proteins required for substrate transport across the bacterial membrane (32). By sequence similarity, both *plpA* and *ami* encode members of the family of permeases responsible for the binding and transport of small peptides (3, 4, 25).

The *ami* locus contains five elements that encode a substrate-binding protein (AmiA) and membrane-associated proteins (AmiC, AmiD, AmiE, and AmiF) that function as the substrate transport machinery (2–4). In contrast, *plpA* does not contain these adjacent downstream elements (2, 25). To determine if the expression of AmiA and deletion of the trans-

TABLE 2. Effects of glycoconjugates on adherence of pneumococci to human vascular EC<sup>a</sup>

Bacterial strain (defective allele)	No. of adherent bacteria/100 eucaryotic cells <sup>b</sup>		
	No addition	GalNAc $\beta$ 1-4Gal	GalNAc $\beta$ 1-3Gal
SPRU98 ( <i>plpA</i> )	130 $\pm$ 15	77 $\pm$ 10*	132 $\pm$ 11
SPRU121 ( <i>amiA</i> )	100 $\pm$ 8	104 $\pm$ 10	53 $\pm$ 9*
SPRU148 ( <i>amiC</i> )	114 $\pm$ 1	109 $\pm$ 11	63 $\pm$ 7*
R6x (none [parent])	240 $\pm$ 10	157 $\pm$ 10*	130 $\pm$ 9*

<sup>a</sup> Similar values were obtained when cultured LC were used.

<sup>b</sup> Carbohydrates chosen were those which best represent receptor populations recognizing pneumococci on resting LC and vascular EC: asialo-GM2 with a terminal GalNAc $\beta$ 1-4Gal moiety and globoside with a terminal GalNAc $\beta$ 1-3Gal moiety (11, 12, 14). Pneumococcal strains ( $2 \times 10^7$  CFU/ml) were incubated with an equal volume of either an albumin-containing buffer (no addition), asialo-GM2 (100  $\mu$ M), or globoside (100  $\mu$ M). Values are the means  $\pm$  standard deviations of six experiments, with the value for each experiment being the mean of results for two replicate wells. \*, significantly less ( $P < 0.05$ ) than adherence of the corresponding control.

port machinery would restore adherence, we constructed a mutant (SPRU148) with an insertion in *amiC* just downstream of *amiA* (25). This mutant was adhesion deficient to the same extent as SPRU121, which contains an insertion in *amiA* (Fig. 1B). Finally, we cannot formally rule out the possibility that the phenotypes observed with these mutants are due to a polar effect of the insertions on elements downstream of the disrupted loci.

**Mutants with defects in *plpA* and *ami* bind to different classes of glycoconjugate receptors.** We have previously described three classes of sugar receptors present on LC and EC that mediate pneumococcal adherence (11, 13). Two glycoconjugate receptors with GalNAc $\beta$ 1-4Gal and GalNAc $\beta$ 1-3Gal specificity combine to mediate the adherence of pneumococci to these eucaryotic cells in a resting state (11, 13). A third GlcNAc-specific glycoconjugate receptor is expressed following cytokine stimulation (11). In competition experiments, the adherence to LC and EC of a mutant with a disruption in *plpA* was further decreased by asialo-GM2 (GalNAc $\beta$ 1-4Gal) but not by globoside (GalNAc $\beta$ 1-3Gal) (Table 2). This finding shows that the effect of the mutation altered the expression of the GalNAc $\beta$ 1-3Gal-specific ligand. In a parallel experiment, a mutant with a disruption in *amiA* showed no decrease in adherence to LC and EC with the addition of asialo-GM2 (GalNAc $\beta$ 1-4Gal), whereas adherence was diminished in the presence of globoside (GalNAc $\beta$ 1-3Gal) (Table 2). This result shows that a mutation in *ami* does not alter the expression of the bacterial ligand specific for the GalNAc $\beta$ 1-3Gal receptor but does reduce the expression of the GalNAc $\beta$ 1-4Gal-specific ligand. All pneumococcal strains tested demonstrated similar percentage increases in adherence following activation of EC by TNF (~70%) and LC by IL-1 $\alpha$  (~30%) (Fig. 2). As a positive control, SPRU30, which has a defined mutation (*glpD*) in a trisaccharide transporter with sequence similarity to the glycerol transporter from *Bacillus subtilis* (26), does not bind to the cytokine-induced receptor and presumably has a defect in the expression of this pneumococcal ligand (unpublished data). In competition experiments, the increased adherence observed with the *ami*- and *plpA*-defective mutants was abrogated by the addition of GlcNAc. At an input concentration of  $10^7$  CFU/ml, levels of adherence to TNF-stimulated EC with an *amiA*-defective mutant were  $141 \pm 13$  and  $224 \pm 16$  adherent bacteria per 100 eucaryotic cells in the presence and absence of 50 mM GlcNAc, respectively. Similar results were obtained with a *plpA*-defective mutant (data not shown). Therefore, the cyto-



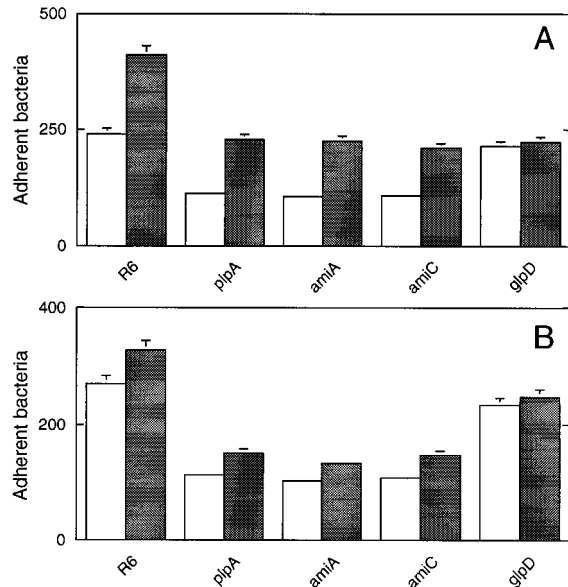


FIG. 2. Adherence of *amiA*-, *amiC*-, and *plpA*-defective mutants to cytokine-stimulated and resting EC and LC. EC monolayers (A) were stimulated with TNF (10 ng/ml, 3 h at 37°C), and LC (B) were incubated with IL-1 (5 ng/ml, 4 h at 37°C). Cytokine-stimulated and resting monolayers were incubated with  $10^8$  CFU of pneumococci per ml for 30 min at 37°C. Permease mutants with defects in *amiA*, *amiC*, or *plpA* were compared with the control strains, R6 (parent) and a mutant with a defect in *glpD* which encodes a putative trisaccharide transporter (26) and does not express the cytokine-induced receptor. Bacteria adherent to resting (open bars) and activated (shaded bars) EC and LC were determined as the number of attached bacteria per 100 eucaryotic cells. The input concentration of bacteria was  $10^7$  CFU/ml. Results are the means  $\pm$  standard deviations for duplicate wells in at least six independent experiments. The decreased values obtained for the permease mutants were statistically significant ( $P < 0.05$ ) compared with values for the resting cells or the *glpD*-defective mutant.

cytokine-stimulated GlcNAc-specific ligand remained intact in these strains.

**Direct binding of adherence-deficient mutants to immobilized glycoconjugates.** To determine if mutations in the permeases decreased pneumococcal binding to the cell-specific sugars, a direct bacterium-glycoconjugate adherence assay was designed. Mutants with defects in *plpA* showed a 60% decrease in adherence to globoside (GalNAc $\beta$ 1-3Gal), while the strains with defects in *amiA* and *amiC* showed decreased adherence to asialo-GM2 (GalNAc $\beta$ 1-4Gal) of 56 and 66%, respectively (Table 3). All mutants adhered to GlcNAc with the same avidity as the parent strain. These results confirm a distinct role for each allele in affecting the expression of different pneumococcal ligands.

## DISCUSSION

As for all bacterial pathogens, pneumococcal adherence to target eucaryotic cells is critical for survival and infection in the human host. Cumulative work from our laboratory and others suggests that at least one class of bacterial ligand is required for nasopharyngeal colonization and that at least three classes of pneumococcal ligands are required for adherence to glycoconjugate receptors on LC and EC (7, 8, 11, 13, 23). Two types of glycoconjugate receptors with specificities for GalNAc $\beta$ 1-3Gal and GalNAc $\beta$ 1-4Gal are present on resting cells, while a third glycoconjugate receptor with a specificity for GlcNAc is induced following cytokine stimulation (11, 13). On the basis of these studies, we have proposed a two-step molecular model for pneumococcal adherence to eucaryotic cells during infection (12). The initial encounter between pneumococci and naive host cells is mediated by resting eucaryotic cell receptors. With increasing numbers of bacteria, an induced inflammatory response up regulates new eucaryotic cell receptors that promote additional bacterial adherence. The increased numbers of bound bacteria at this stage may be responsible for the localized tissue damage observed with pneumococcal infection. Results from our laboratory have shown that cytokine activation of both LC and EC leads to the de novo appearance of the platelet-activating factor receptor, which serves as the induced receptor for the pneumococci with a specificity for GlcNAc (11). The up regulation of this new receptor is corroborated by another recent study that demonstrated increased pneumococcal adherence to LC following infection with adenovirus (19).

Exported proteins, important to the survival of bacterial pathogens, not only maintain normal cellular functions but also serve as determinants of virulence, mediating adherence and colonization of cell surfaces or protecting the bacteria against the host's immune response. Recently our laboratory developed a genetic strategy based on translational fusions to a bacterial gene for alkaline phosphatase to create pneumococcal mutants with defects in exported proteins (26). By assessing these mutants for loss of function in in vitro adherence assays, we have identified bacterial elements that affect this process and potentially reflect similar requirements in vivo during infection. With this strategy, three independent mutants with alterations in either of two previously described loci, *plpA* and *ami*, demonstrated a 50 to 60% decrease in adherence to the GalNAc $\beta$ 1-3Gal and GalNAc $\beta$ 1-4Gal receptors, respectively, present on resting LC and EC. Compared with the parent strain, these mutants agglutinated neuraminidase-treated erythrocytes in a process mediated by sugar receptors (GlcNAc $\beta$ 1-4Gal) similar to those found on human buccal epithelial cells. These mutants also bound the GlcNAc-specific receptor present on cytokine-stimulated cells. These results were confirmed with the decreased binding of each mutant to ana-

TABLE 3. Direct adherence of pneumococci to immobilized glycoconjugates

Bacterial strain (altered allele)	No. of adherent bacteria in a 40 $\times$ microscope field <sup>a</sup>			
	No sugar	GalNAc $\beta$ 1-4Gal	GalNAc $\beta$ 1-3Gal	GlcNAc
SPRU98 ( <i>plpA</i> )	54 $\pm$ 15	384 $\pm$ 12	70 $\pm$ 11*	123 $\pm$ 10
SPRU121 ( <i>amiA</i> )	40 $\pm$ 8	140 $\pm$ 13*	180 $\pm$ 11	124 $\pm$ 11
SPRU148 ( <i>amiC</i> )	54 $\pm$ 12	124 $\pm$ 10*	164 $\pm$ 7	131 $\pm$ 13
R6x (none [parent])	60 $\pm$ 10	370 $\pm$ 10	175 $\pm$ 9	118 $\pm$ 12

<sup>a</sup> Pneumococci ( $10^7$  CFU/ml) were incubated with immobilized asialo-GM2 (100  $\mu$ M), globoside (100  $\mu$ M), or GlcNAc (50 mM) for 30 min at 37°C. Values are the means  $\pm$  standard deviations of six experiments, with the value for each experiment being the mean of results for two replicate wells. Carbohydrates chosen were those which best represent receptor populations recognizing pneumococci on either resting (GalNAc $\beta$ 1-4Gal [asialo-GM2] and GalNAc $\beta$ 1-3Gal [globoside]) or cytokine-activated (GlcNAc) LC and vascular EC (11, 12, 14). \*, significantly less ( $P < 0.05$ ) than the value obtained with the parent strain R6 and the corresponding sugar.

logs of the proposed cognate glycoconjugate receptors on these cells. We conclude that mutations in *plpA* and *ami* loci modulate the process of adherence to two specific glycoconjugate receptors on host target cells.

We have isolated other mutants which express decreased adherence to the different eucaryotic receptors. One mutant with a defect in pyruvate oxidase which leads to decreased production of the intracellular messenger acetyl phosphate has a global loss in the expression of all of the pneumococcal ligands (37). Another mutant with a defect in a member of the carbohydrate:phosphoenolpyruvate transfer system showed reduced binding only to the GlcNAc-specific receptor (unpublished data). Mutants with defects in a locus that includes *glpD* (Fig. 2) and is responsible for trisaccharide transport and opacity variation in colony morphology are also defective in binding to the GlcNAc-specific receptor (14, 26, 33). Therefore, this mutagenesis and screening strategy demonstrates several classes of ligands present on the surface of pneumococci affected by a variety of elements.

Three distinct peptide-binding protein-dependent permeases have been identified in pneumococci (2–4, 25). Experimental evidence suggests that they, like all members of this family, bind and transport small peptides across the bacterial membrane presumably to scavenge these molecules for metabolic utilization (2, 36). There is now a body of evidence that suggests that this class of permeases from both gram-negative and gram-positive organisms modulates through cell signaling several bacterial processes such as chemotaxis in *Salmonella typhimurium* and *E. coli* (1, 24), pheromone-mediated conjugation in *Enterococcus faecalis* (30, 40), and sporulation and natural transformation in *B. subtilis* (27, 29). Work from our laboratory (25) and confirmed by others (2) has shown that mutations in *plpA* down regulate the process of natural transformation, while mutations in the *ami* locus increase the efficiency of this process (25). These results suggest that the binding proteins PlpA and AmiA, which have 60% sequence identity (25), function as distinct regulatory elements to modulate the process of transformation by mediating the binding and/or transport of signaling molecules (25). This current study shows that mutations in *plpA* and *ami* also modulate the expression of two separate ligands required for adherence to human LC and vascular EC. Mutations in *plpA* resulted in decreased binding to the GalNAc $\beta$ 1-3Gal receptor, whereas mutations in the *ami* locus resulted in decreased binding to the GalNAc $\beta$ 1-4Gal receptor. Therefore, we propose that these related permeases mediate distinct adherence properties of pneumococci through the binding and transport of signaling molecules that in turn regulate the expression of pneumococcal ligands. These studies cannot eliminate the formal possibility that these permeases are the structural molecules that directly bind to the eucaryotic cells. Since recent structural studies have shown that OppA, a homolog from *E. coli*, binds peptides somewhat indiscriminately (39), it is possible that these peptide-binding proteins bind glycoprotein receptors on the surface of the eucaryotic cell. A mutation in *amiC* that disrupts production of the peptide transport machinery but that does not alter the expression of AmiA produced a strain that was still adhesion deficient, which therefore argues against this interpretation.

Other permeases present in the viridans group of oral streptococci mediate bacterial coaggregation and adherence to the growing pellicle. SarA from *Streptococcus gordonii*, close to 60% identical to PlpA and AmiA, belongs to the same family of gram-positive peptide-binding proteins (25). Targeted mutagenesis of *sarA* has a global effect on the expression of exported proteins and a dramatic effect on adherence and coaggregation

(20, 21). A sugar-binding permease (MsmE) may mediate the adherence properties of *Streptococcus mutans* (31, 38). Members of a family of putative permeases of unknown substrate specificity from *Streptococcus parasanguis* (FimA [15]), *Streptococcus sanguis* (SsaB [16]), and *S. gordonii* (ScaA [22]) have been shown either to mediate adherence or to be adhesins associated with these bacteria. Sequence analysis of the corresponding genetic loci of these permeases predicts lipid-anchored 35-kDa substrate-binding proteins with greater than 80% sequence identity with a typical complement of membrane-associated proteins that serve a transport machinery. A homolog of these permeases, PsaA, has been identified in pneumococci (35), but a function associated with adherence has yet to be determined. These cumulative data suggest that distinct permeases in related streptococcal species affect, either directly or indirectly, bacterial attachment to various substrates within the target host. Therefore, at least three diverse functions have now been ascribed to the peptide-binding permeases in pneumococci. From the results of targeted mutagenesis and in vitro bioassays, we conclude that these permeases modulate the process of natural transformation (2, 25), adherence to eucaryotic cells (this study), and the binding and transport of small peptides presumably for metabolic utilization (2).

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#### REFERENCES

1. Abouhamad, W. N., M. Manson, M. M. Gibson, and C. F. Higgins. 1991. Peptide transport and chemotaxis in *Escherichia coli* and *Salmonella typhimurium*: characterization of the dipeptide permease (Dpp) and the dipeptide-binding protein. *Mol. Microbiol.* 5:1035–1047.
2. Alloing, G., P. de Philip, and J. P. Claverys. 1994. Three highly homologous membrane-bound lipoproteins participate in oligopeptide transport by the Ami system of the gram-positive *Streptococcus pneumoniae*. *J. Mol. Biol.* 241:44–58.
3. Alloing, G., M. C. Trombe, and J. P. Claverys. 1990. The *ami* locus of the Gram-positive bacterium *Streptococcus pneumoniae* is similar to binding protein-dependent transport operons of Gram-negative bacteria. *Mol. Microbiol.* 4:633–644.
4. Alloing, G., M. C. Trombe, and J. P. Claverys. 1989. Cloning of the *amiA* locus of *Streptococcus pneumoniae* and identification of its functional limits. *Gene* 76:363–368.
5. Andersson, B., T. Dahmen, T. Freijid, H. Leffler, G. Magnusson, G. Noori, and C. Svanborg-Eden. 1983. Identification of an active disaccharide unit of a glycoconjugate receptor of pneumococci attaching to human pharyngeal epithelial cells. *J. Exp. Med.* 158:559–570.
6. Andersson, B., B. Ericksson, E. Falsen, A. Fogh, L. Å. Hanson, O. Nylén, H. Peterson, and C. Svanborg-Edén. 1981. Adhesion of *Streptococcus pneumoniae* to human pharyngeal epithelial cells in vitro: differences in adhesive capacity among strains isolated from subjects with otitis media, septicemia, or meningitis or from healthy carriers. *Infect. Immun.* 32:311–317.
7. Andersson, B., B. Gray, and C. S. Eden. 1988. Role of attachment for the virulence of *Streptococcus pneumoniae*. *Acta Oto-Laryngol. Suppl.* 454:163–166.
8. Andersson, B., and C. Svanborg Eden. 1988. Mechanisms of pneumococcal attachment. *Monogr. Allergy* 24:44–45.
9. Austrian, R. 1986. Some aspects of the pneumococcal carrier state. *J. Antimicrob. Chemother.* 18:S35–S45.
10. Chen, J. D., and D. A. Morrison. 1988. Construction and properties of a new insertion vector, pJDC9, that is protected by transcriptional terminators and useful for cloning of DNA from *Streptococcus pneumoniae*. *Gene* 64:155–164.
11. Cundell, D. R., N. P. Gerard, C. Gerard, and E. I. Tuomanen. Platelet activating factor receptor anchors *Streptococcus pneumoniae* to activated human cells. Submitted for publication.
12. Cundell, D. R., H. R. Masure, and E. I. Tuomanen. The pathogenesis of pneumococcal disease. A molecular model. *Clin. Infect. Dis.*, in press.
13. Cundell, D. R., and E. I. Tuomanen. Receptor specificity of adherence of *Streptococcus pneumoniae* to human type II pneumocytes and vascular en-

- dothelial cells *in vitro*. *Microb. Pathog.* **17**:361–374.
14. Cundell, D. R., J. N. Weiser, A. Young, and E. I. Tuomanen. 1995. Relationship between colonial morphology and adherence in *Streptococcus pneumoniae*. *Infect. Immun.* **63**:757–761.
  15. Fenno, J. C., D. J. LeBlanc, and P. Fives-Taylor. 1989. Nucleotide sequence analysis of type 1 fimbrial gene of *Streptococcus sanguis* FW213. *Infect. Immun.* **57**:3527–3533.
  16. Ganeshkumar, N., P. M. Hannam, P. E. Kolenbrander, and B. C. McBride. 1991. Nucleotide sequence of a gene coding for a saliva-binding protein (SsaB) from *Streptococcus sanguis* 12 and possible role of the protein in coaggregation with actinomycetes. *Infect. Immun.* **59**:1093–1099.
  17. Geelen, S., C. Bhattacharyya, and E. Tuomanen. 1992. Induction of procoagulant activity on human endothelial cells by *Streptococcus pneumoniae*. *Infect. Immun.* **60**:4179–4183.
  18. Geelen, S., C. Bhattacharyya, and E. Tuomanen. 1993. The cell wall mediates pneumococcal attachment to and cytopathology in human endothelial cells. *Infect. Immun.* **61**:1538–1543.
  19. Hakansson, A., A. Kidd, G. Wadell, H. Sabharwal, and C. Svanborg. 1994. Adenovirus infection enhances *in vitro* adherence of *Streptococcus pneumoniae*. *Infect. Immun.* **62**:2707–2714.
  20. Jenkinson, H. F. 1992. Adherence, coaggregation, and hydrophobicity of *Streptococcus gordonii* associated with expression of cell surface lipoproteins. *Infect. Immun.* **60**:1225–1228.
  21. Jenkinson, H. F., and R. A. Easingwood. 1990. Insertional inactivation of the gene encoding a 76-kilodalton cell surface polypeptide in *Streptococcus gordonii* Challis has a pleiotropic effect on cell surface composition and properties. *Infect. Immun.* **58**:3689–3697.
  22. Kolenbrander, P. E., R. N. Andersen, and N. Ganeshkumar. 1994. Nucleotide sequence of the *Streptococcus gordonii* PK488 coaggregation adhesin gene, *scaA*, and ATP-binding cassette. *Infect. Immun.* **62**:4469–4480.
  23. Krivan, H. C., D. D. Roberts, and V. Ginsberg. 1988. Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc $\beta$ 1-4Gal found in some glycolipids. *Proc. Natl. Acad. Sci. USA* **85**:6157–6161.
  24. Manson, M. D., V. Blank, G. Brade, and C. R. Higgins. 1986. Peptide chemotaxis in *E. coli* involves the Tap signal transducer and the dipeptide permease. *Nature (London)* **321**:253–256.
  25. Pearce, B. J., A. M. Naughton, and H. R. Masure. 1994. Peptide permeases modulate transformation in *Streptococcus pneumoniae*. *Mol. Microbiol.* **12**:881–892.
  26. Pearce, B. J., Y. B. Yin, and H. R. Masure. 1993. Genetic identification of exported proteins in *Streptococcus pneumoniae*. *Mol. Microbiol.* **9**:1037–1050.
  27. Perego, M., C. F. Higgins, S. R. Pearce, M. P. Gallagher, and J. A. Hoch. 1991. The oligopeptide transport system of *Bacillus subtilis* plays a role in the initiation of sporulation. *Mol. Microbiol.* **5**:173–185.
  28. Rake, G. 1936. Pathogenesis of pneumococcus infection in mice following intranasal instillation. *J. Exp. Med.* **63**:17–37.
  29. Rudner, D. Z., J. R. LeDeaux, K. Ireton, and A. D. Grossman. 1991. The *spo0K* locus of *Bacillus subtilis* is homologous to the oligopeptide permease locus and is required for sporulation and competence. *J. Bacteriol.* **173**:1388–1398.
  30. Ruhfel, R. E., D. A. Manias, and G. M. Dunny. 1993. Cloning and characterization of a region of the *Enterococcus faecalis* conjugative plasmid, pCF10, encoding a sex pheromone-binding function. *J. Bacteriol.* **175**:5253–5259.
  31. Russell, R. R., O. J. Aduse, I. C. Sutcliffe, L. Tao, and J. J. Ferretti. 1992. A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolism. *J. Biol. Chem.* **267**:4631–4637.
  32. Saier, M. H. 1994. Computer-aided analyses of transport protein sequences: gleaned evidence concerning function, structure, biogenesis, and evolution. *Microbiol. Rev.* **58**:71–93.
  33. Saluja, S., and J. N. Weiser. The genetic basis of colonial opacity in *Streptococcus pneumoniae*: evidence for the effect of the box elements on phenotypic variation. *Mol. Microbiol.*, in press.
  34. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
  35. Sampson, J. S., S. P. O'Connor, A. R. Stinson, J. A. Tharpe, and H. Russell. 1994. Cloning and nucleotide sequence analysis of *psaA*, the *Streptococcus pneumoniae* gene encoding a 37-kilodalton protein homologous to previously reported *Streptococcus* sp. adhesins. *Infect. Immun.* **62**:319–324.
  36. Sicard, A. M. 1964. A new synthetic medium for *Diplococcus pneumoniae*, and its use for the study of reciprocal transformations at the *amiA* locus. *Genetics* **50**:31–44.
  37. Spellerberg, B., J. Sandros, D. R. Cundell, B. J. Pearce, and H. R. Masure. Global regulation of adherence as a determinant for virulence of *Streptococcus pneumoniae*. Submitted for publication.
  38. Sutcliffe, I. C., L. Tao, J. J. Ferretti, and R. R. Russell. 1993. MsmE, a lipoprotein involved in sugar transport in *Streptococcus mutans*. *J. Bacteriol.* **175**:1853–1855.
  39. Tame, J. R. H., G. N. Murshudov, T. K. Neil, E. J. Dodson, C. F. Higgins, and A. J. Wilkinson. 1994. The structural basis of sequence-independent peptide binding by OppA protein. *Science* **264**:1578–1581.
  40. Tanimoto, K., F. Y. An, and D. B. Clewell. 1993. Characterization of the *traC* determinant of the *Enterococcus faecalis* hemolysin-bacteriocin plasmid pAD1: binding of sex pheromone. *J. Bacteriol.* **175**:5260–5264.
  41. Tiraby, G., and M. S. Fox. 1973. Marker discrimination in transformation and mutation of pneumococcus. *Proc. Natl. Acad. Sci. USA* **70**:3541–3545.
  42. Tuomanen, E. I., R. Austrian, and H. R. Masure. The pathogenesis of pneumococcal infection: correlation of clinical events with molecular mechanisms. *N. Engl. J. Med.*, in press.
  43. Wood, W. B. 1951. Studies on the cellular immunology of acute bacterial infections. *Harvey Lect.* **47**:72–98.
  44. Yother, J., and D. E. Briles. 1992. Structural properties and evolutionary relationships of PspA, a surface protein of *Streptococcus pneumoniae*, as revealed by sequence analysis. *J. Bacteriol.* **174**:601–609.
  45. Yother, J., G. L. Handsome, and D. E. Briles. 1992. Truncated forms of PspA that are secreted from *Streptococcus pneumoniae* and their use in functional studies and cloning of the *pspA* gene. *J. Bacteriol.* **174**:610–618.