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The Vaccine Candidate Substrate Binding Protein SBP2 Plays a Key Role in Arginine Uptake, Which Is Required for Growth of *Moraxella catarrhalis*

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***Moraxella catarrhalis* is an exclusively human pathogen that is an important cause of otitis media in children and lower respiratory tract infections in adults with chronic obstructive pulmonary disease. A vaccine to prevent *M. catarrhalis* infections would have an enormous global impact in reducing morbidity resulting from these infections. Substrate binding protein 2 (SBP2) of an ABC transporter system has recently been identified as a promising vaccine candidate antigen on the bacterial surface of *M. catarrhalis*. In this study, we showed that SBP1, -2, and -3 individually bind different basic amino acids with exquisite specificity. We engineered mutants that each expressed a single SBP from this gene cluster and showed in growth experiments that SBP1, -2, and -3 serve a nutritional function through acquisition of amino acids for the bacterium. SBP2 mediates uptake of arginine, a strict growth requirement of *M. catarrhalis*. Adherence and invasion assays demonstrated that SBP1 and SBP3 play a role in invasion of human respiratory epithelial cells, consistent with a nutritional role in intracellular survival in the human respiratory tract. This work demonstrates that the SBPs of an ABC transporter system function in the uptake of basic amino acids to support growth of *M. catarrhalis*. The critical role of SBP2 in arginine uptake may contribute to its potential as a vaccine antigen.**

Moraxella catarrhalis is an exclusively human pathogen whose ecological niche is the human respiratory tract. Nasopharyngeal colonization by *M. catarrhalis* is common in infancy and decreases with age (1–5). The bacterium is a common cause of disease in two clinical settings: otitis media in children and lower respiratory tract infection in adults with chronic obstructive pulmonary disease (COPD) (6–8).

Approximately 80% of all children experience at least one episode of otitis media (middle ear infection) by the age of 3 years (9). One recent study showed that up to 5% of pediatric outpatients, even without symptoms, had acute otitis media (10). Up to 30% of children develop recurrent acute otitis media (11–13), which may lead to hearing impairment that causes delays in speech and language development (14). A vaccine to prevent these infections would have a major impact on child health by preventing morbidity and reducing health care costs associated with otitis media. *M. catarrhalis* is a common cause of acute otitis media in children, along with nontypeable *Haemophilus influenzae* and *Streptococcus pneumoniae* (6). Studies using PCR to detect bacterial DNA in middle ear fluid reveal that *M. catarrhalis* plays an even larger role in otitis media than revealed by culture alone (15–18). With the increasing widespread administration of pneumococcal conjugate vaccines globally, patterns of nasopharyngeal colonization in children and the distribution of pathogens causing otitis media are undergoing changes, with *M. catarrhalis* taking on a more prominent role (19). Thus, a successful vaccine for otitis media will include antigens of the three otopathogens, *M. catarrhalis*, nontypeable *H. influenzae*, and *S. pneumoniae*.

M. catarrhalis is also an important pathogen in adults with COPD, which is a chronic debilitating disease. COPD is the third most common cause of death in the United States and the world. The course of COPD is characterized by intermittent worsening or exacerbations, often caused by bacterial infection of the lower airways (20). *M. catarrhalis* is the second most common cause of exacerbations, accounting for 2 to 4 million episodes annually in the United States (21). Thus, adults with COPD would also benefit from a vaccine to prevent respiratory tract infections caused by *M. catarrhalis*.

In view of the compelling public health need for effective methods to prevent otitis media in children and lower respiratory tract infections in adults with COPD, several groups are performing research to develop vaccines to prevent *M. catarrhalis* infections. We have taken a genome-mining approach to identify conserved proteins that are on the bacterial surface that we are assessing as potential vaccine antigens (22). This work has led to the identification of several surface proteins that induce potentially protective immune responses to *M. catarrhalis* (23–25).

One such protein, substrate binding protein 2 (SBP2), is predicted to be one of three substrate binding proteins of an ATP-binding cassette (ABC) transporter system encoded by a gene cluster annotated in the *M. catarrhalis* genome (25). SBP2 is highly conserved among strains, expresses epitopes on the bacterial sur-

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face, and induces potentially protective immune responses in the mouse pulmonary clearance model; thus, SBP2 is a promising vaccine antigen. In view of the importance of SBP2 as a vaccine antigen, the present study was undertaken to characterize the function of the ABC transporter and its three substrate binding proteins (SBP1, SBP2, and SBP3) for the bacterial cell and to begin to understand the role of the proteins in the pathogenesis of *M. catarrhalis* infection. The ABC transporter gene cluster encodes three SBPs that are homologous with amino acid transport substrate binding proteins of other Gram-negative bacteria in the range of 50% identity and 70% similarity (25). In the present study, we show that the gene cluster encodes proteins that individually bind basic amino acids specifically and have a nutritional function through acquisition of essential amino acids for the bacterium. SBP2 mediates uptake of arginine, a strict growth requirement of *M. catarrhalis*. In addition, at least two of the three substrate binding proteins play a critical role in invasion of human respiratory epithelial cells.

MATERIALS AND METHODS

Bacterial strains and growth. *M. catarrhalis* strain O35E was provided by Eric Hansen. Single-gene (*sbp1*, *sbp2*, and *sbp3*) knockout mutants and a mutant in which all three *sbp* genes were knocked out (*sbp123*) were previously constructed in *M. catarrhalis* strain O35E (25). *M. catarrhalis* strains were grown on brain heart infusion (BHI) plates at 35°C with 5% CO_2 or in BHI broth with shaking at 37°C. For growth curve experiments, chemically defined medium (CDM), which is a minimal nutrient medium containing the essential elements that *M. catarrhalis* requires to survive, was used (26).

Purification of recombinant SBP1, SBP2, and SBP3. The *sbp1*, *sbp2*, and *sbp3* genes were individually cloned into the plasmid pCATCH which allows expression of recombinant lipoproteins in *Escherichia coli* (27, 28). Purification of the SBP1, SBP2, and SBP3 proteins and assessment of their purity have been described in detail previously (25).

Thermal shift assay. Thermal shift assays were performed using a Stratagene Mx3005P real-time PCR instrument (Stratagene, La Jolla, CA) as previously described (29, 30). Briefly, purified SBP1, SBP2, and SBP3 were studied at a concentration of 10 μg in a 30-μl volume in buffer (0.01 M Tris, 0.15 M NaCl, pH 7.4) to which individual amino acids were added to a final concentration of 1 mM. SYPRO Orange (Sigma) was added as a fluorescence reporter at a 1,000-fold dilution from its stock solution. The change in fluorescence was monitored using a Cy3 filter, with excitation and emission wavelengths of 545 nM and 568 nM, respectively. Temperature was raised from 25°C to 98°C in 0.5°C intervals over the course of 45 min, and fluorescence readings were taken at each interval. The fluorescence data were plotted and normalized, and the first derivative of the curve was calculated to provide the melting temperatures (T_ms) using GraphPad Prism, version 5.0, as previously described (29, 31).

Construction of mutants. New mutants were constructed in *M. catarrhalis* strain O35E in which two of the three genes that encode substrate binding proteins were knocked out so that each new mutant expressed a single SBP. For example, knocking out the *sbp1* and *sbp2* genes leaves the *sbp3* gene which expresses SBP3 as the only substrate binding protein expressed from the gene cluster. Similar mutants that express SBP1 and SBP2 individually were engineered by knocking out the *sbp2* and *sbp3* genes and the *sbp1* and *sbp3* gene, respectively.

Mutant construction was accomplished by using overlap extension PCR and homologous recombination as described previously (24, 25, 32). Briefly, the transforming DNA for the mutants was composed of three overlapping fragments that included 1 kb upstream of the gene being knocked out (fragment 1), the nonpolar kanamycin resistance cassette amplified from plasmid pUC18K (33) (fragment 2), and 1 kb downstream of the gene (fragment 3) using the oligonucleotide primers listed in Table S1 in the supplemental material. Mutants were constructed by transformation of strain O35E with a fragment composed of fragments 1, 2, and 3 and selection on BHI plates containing 50 μg/ml of kanamycin. The *sbp1 sbp3* mutant was constructed in an *sbp1* gene mutant (25) using the spectinomycin resistance cassette amplified from plasmid pSPEC1 as fragment 2 (34). The insert and surrounding sequences of each of the mutants were confirmed by sequence analysis.

Assessment of bacterial growth. Growth curves were performed using a Bioscreen C automated growth curve analysis system (Oy Growth Curves AB Ltd., Helsinki, Finland) according to the manufacturer's instructions. For *M. catarrhalis* strains, readings of the optical density at 600 nm (OD_{600}) were taken at 30-min intervals with the Bioscreen C system at 37°C with constant shaking (machine settings: fast speed and high amplitude). Five replicate wells were performed for each growth condition. CDM with or without specific amino acid(s) was used for these experiments.

Adherence and invasion assays with respiratory epithelial cells. Quantitative adherence and invasion assays were performed with A549 cells (human type II alveolar lung epithelium; ATCC CCL85) grown in F-12K medium (Gibco, Grand Island, NY) plus 10% fetal bovine serum. Briefly, 2×10^5 A549 cells were seeded into each well of a 24-well tissue culture plate and incubated for ~48 h, when cells showed confluent growth. Cells were inoculated with broth-grown log-phase bacteria (multiplicity of infection of 1), and the plates were centrifuged at $200 \times g$ for 5 min at room temperature to facilitate contact between bacteria and A549 cells. Plates were incubated for 3 h at 37°C in 5% CO_2. Nonadherent cells were removed by gently washing the wells three times with 500 μl of phosphate-buffered saline (PBS). To quantify adherent cells, 200 μl of trypsin (0.25%) was added to each well, and plates were incubated at 37°C for 10 min to remove adherent cells. A 300-μl volume of 1% saponin was applied to each well, and contents were pipetted into microcentrifuge tubes and, after vigorous vortexing, plated in duplicate to perform bacterial cell counts of adherent cells. Medium from infected samples was harvested, diluted in PBS, and cultured on chocolate agar. The colony counts from medium and from cells were added together to calculate the total number of CFU in a sample well. Percent adherence was calculated by dividing the number of CFU per milliliter of adherent bacteria by the number of CFU per milliliter of the total bacteria in the well. Results of assays with mutants were expressed as a percentage of the result with the wild type that was assayed simultaneously. Each experiment was repeated three times.

To measure invasion, gentamicin (100 μg/ml) was added to wells after 3 h of incubation of A549 cells with bacteria. Nonadherent cells were removed by washing, and wells were incubated with gentamicin for 1 h at 37°C in 5% CO_2 to kill extracellular bacteria. Cells were removed with trypsin and lysed with saponin as described above to release surviving intracellular bacteria, which were then plated in duplicate. Percent invasion was calculated by dividing the number of CFU per milliliter of intracellular bacteria by the number of CFU per milliliter of the total bacteria in the well. Results of assays with mutants were expressed as a percentage of the result with the wild type that was assayed simultaneously. Each experiment was repeated three times. Statistical significance was determined by performing a two-tailed *t* test comparing the result with each mutant to that with the wild type.

RESULTS

Binding of amino acids to the SBPs. Thermal shift assays were used to assess the binding of amino acids to purified recombinant SBP1, SBP2, and SBP3. All three proteins showed sharp T_ms in buffer in the absence of added amino acids, indicating that the proteins formed a stable conformation (for SBP1, T_m of 59.6°C; for SBP2, T_m of 57.6°C; for SBP3, T_m of 60.7°C) (Table 1). Addition of each of 21 amino acids (20 standard amino acids plus ornithine) individually to the purified proteins and measurement of thermal shifts showed striking specificity in the binding of selected amino acids as indicated by thermal shifts (Table 1). SBP1

TABLE 1 Melting temperatures and results of thermal shift assays with purified SBP1, SBP2, and SBP3

Amino acid	SBP1		SBP2		SBP3	
	T_m (°C)[a]	ΔT_m (°C)[b]	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)
None (buffer only)	59.6		57.6		60.7	
Alanine	58.9	−0.7	57.6	0.0	60.8	0.1
Cysteine	58.0	−1.6	57.8	0.2	61.3	0.6
Aspartic acid	60.9	1.3	57.7	0.1	60.8	0.1
Glutamic acid	59.4	−0.2	57.7	0.1	60.7	0.0
Phenylalanine	60.8	1.2	57.7	0.1	60.9	0.2
Glycine	59.2	−0.4	57.8	0.2	60.8	0.1
Histidine	58.2	−1.4	58.3	0.7	60.9	0.2
Isoleucine	58.8	−0.8	57.8	0.2	60.7	0.0
Lysine	**71.4**	**11.8**	57.5	−0.1	62.3	1.6
Leucine	58.8	−0.8	57.6	0.0	60.3	−0.4
Methionine	58.9	−0.7	57.7	0.1	60.9	0.2
Asparagine	59.1	−0.5	57.8	0.2	60.7	0.0
Ornithine	59.5	−0.1	56.7	−0.9	**69.4**	**8.7**
Proline	59.8	0.2	56.8	−0.8	60.5	−0.2
Glutamine	59.1	−0.5	56.8	−0.8	60.2	−0.5
Arginine	58.8	−0.8	**64.6**	**7.0**	61.3	0.6
Serine	58.6	−1.0	57.6	0.0	60.7	0.0
Threonine	59.3	−0.3	57.7	0.1	60.5	−0.2
Valine	59.8	0.2	57.8	0.2	60.3	−0.4
Tryptophan	59.6	0.0	57.9	0.3	60.5	−0.2
Tyrosine	58.9	−0.7	57.4	−0.2	60.6	−0.1

[a] T_m, melting temperature.
[b] ΔT_m, thermal shift. Assays showing thermal shifts are noted in bold.

showed an upward shift of the T_m of 11.8°C with lysine and no shift with other amino acids. SBP2 showed a shift of 7.0°C with arginine and no shift with other amino acids. SBP3 showed a shift of 8.7°C with ornithine and no shift with other amino acids. We conclude that SBP1 binds lysine, SBP2 binds arginine, and SBP3 binds ornithine with high specificity.

Characterization of expression of SBPs in knockout mutants. To assess the role of each of the three SBPs in the gene cluster under study, mutants that expressed a single SBP in the gene cluster were constructed and characterized. Mutants in which two of the three *sbp* genes were knocked out each expressed a single SBP. The *sbp23* knockout mutant expresses SBP1, the *sbp13* knockout mutant expresses SPB2, and the *sbp12* mutant expresses SBP3 (Fig. 1). Immunoblot assays with antiserum to each of the purified SBPs show that antiserum raised to the purified protein recognizes its corresponding SBP and that the new mutants express a single SBP (Fig. 1).

Role of SBPs in bacterial growth. In order to assess the function of SBPs in the uptake of amino acids for growth of *M. catarrhalis*, we studied the mutants that individually expressed each one of the three SBPs. For example, a mutant in which *spb2* and *sbp3* were knocked out expressed only SBP1 and so forth. Arginine is essential for *M. catarrhalis* growth but can be replaced with ornithine, which is a downstream by-product of arginine metabolism (26, 35). Thermal shift assays demonstrated the primary binding specificities of each of the three proteins: SBP1 binds lysine, SBP2 binds arginine, and SBP3 binds ornithine (Table 1). Table 2 and Fig. S1 in the supplemental material show the results of growth curve experiments with wild-type and mutant strains in CDM that excluded or was supplemented with specific amino acids.

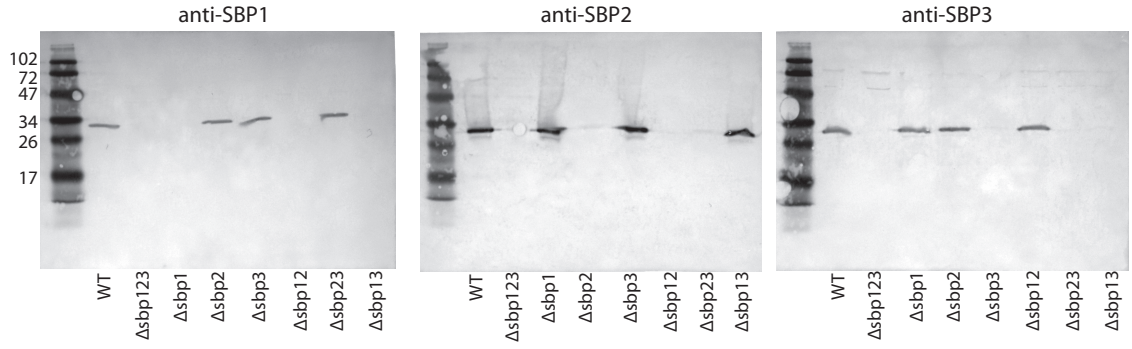

FIG 1 Immunoblot assays of whole-cell lysates of wild-type (WT) and mutant strains as noted for each lane at the bottom of the blots. Immunoblots were probed with antisera raised to purified recombinant SBPs as noted at the top of each panel. Molecular mass markers are noted in kilodaltons on the left. Note that *sbp1*, *sbp2*, *sbp3*, and *sbp123* mutants were described by Otsuka et al. (25); they are included here to show each mutant used in the present study.

TABLE 2 Growth characteristics of O35E and *sbp* mutants in chemically defined medium

Strain	Expression of SBP(s)	Growth on CDM with specific amino acid content[a]						Affinity of SBP for amino acid uptake
		Arg^+ Lys^+ Orn^-	Arg^+ Lys^- Orn^-	Arg^- Lys^+ Orn^+	Arg^- Lys^- Orn^+	Arg^+ Lys^+ Orn^+	Arg^- Lys^+ Orn^-	
O35E	SBP1, -2, -3	3+	3+	1+	2+	3+	−	Arg, Lys, Orn
sbp23 mutant	SBP1	2+	3+	−	1+	2+	−	Lys ≥ Arg > Orn
sbp13 mutant	SBP2	2+	2+	−	−	2+	−	Arg
sbp12 mutant	SBP3	−	1+	2+	2+	2+	−	Orn > Lys > Arg
sbp123 mutant	None	−	−	−	−	−	−	None

[a] Amino acid presence (+ superscript) or absence (− superscript) in the medium is indicated. Growth data represent growth curve peaks as follows: 3+, growth curve peak at ≤16 h; 2+, growth curve peak at 16 h to 24 h; 1+, growth curve peak at ≥24 h; −, no growth.

Role of SBP1 in bacterial growth. Growth curves with the *sbp23* mutant (expresses SBP1) show that SBP1 has binding affinity for uptake of the three basic amino acids in the order of lysine > arginine > ornithine, based on the following observations (Table 2; see also Fig. S1 in the supplemental material). The *sbp123* mutant does not grow in CDM. The *sbp23* mutant requires either arginine or ornithine for growth, indicating that SBP1, which has high affinity for lysine, also has the capacity to mediate uptake of arginine and ornithine. However, in medium that contains both lysine and arginine, growth is slower than that of the wild type, consistent with SBP1 binding lysine (not required for growth) with higher affinity than arginine (required for growth). In medium that lacks arginine but contains both lysine and ornithine, the mutant does not grow at all, consistent with SBP1 binding lysine and completely inhibiting binding of ornithine by SBP1. Thus, these results support the conclusion that SBP1 shows binding affinity for uptake of the three amino acids as follows: lysine > arginine > ornithine (Table 2).

Role of SBP2 in bacterial growth. The *sbp13* mutant (expresses SBP2) requires arginine in CDM for growth (Table 2; see also Fig. S1 in the supplemental material). Ornithine does not substitute for arginine to support the growth of this mutant, indicating that SBP2 does not mediate the uptake of ornithine. We conclude that SBP2 has binding affinity for arginine uptake but not for ornithine uptake.

Role of SBP3 in bacterial growth. Growth curves for the *sbp12* mutant (expresses SBP3) show that SBP3 has affinity for uptake of the three basic amino acids in the order of ornithine > lysine > arginine, based on the following observations. The *sbp12* mutant requires ornithine for growth with one exception. Arginine can partially substitute for ornithine as long as there is no lysine in the medium. The *sbp12* mutant does not grow in the absence of ornithine when lysine and arginine are present together, consistent with SBP3 having a higher affinity for lysine than for arginine. Thus, we conclude that SBP3 shows binding affinity for uptake of the three amino acids as follows: ornithine > lysine > arginine.

The shapes of growth curves when ornithine is present in the absence of arginine differ from the shapes of the curves in the presence of arginine, even for the wild-type strain (see Fig. S1 in the supplemental material). The culture is slower to reach maximum OD and reaches a higher OD with ornithine present in place of arginine. We speculate that the altered growth pattern is related to the activation of different metabolic pathways required for the processing of ornithine compared to those required for arginine.

Role of SBPs in adherence and invasion of human respiratory epithelial cells. Wild-type and SBP mutant strains were assayed for their capacity to adhere to and invade the human respiratory epithelial cell line A549 (type 2 pneumocytes). In performing the assays, we observed that growth of the *sbp2* and *sbp123* knockout mutants was impaired under the conditions of this assay, resulting in ~2-log difference between the mutants and wild type in the number of bacteria in the wells after 3 h. This growth defect is likely due to the critical role that SBP2 plays in uptake of arginine by *M. catarrhalis* and the differences in relative abundances of nutrients in bacterial medium and cell culture medium. Thus, it was not possible to accurately determine adherence and invasion of the *sbp2* and *sbp123* mutants as a result of this confounding variable.

There was no difference in adherence of the *sbp1* and *sbp3* knockout mutants and the wild type to respiratory epithelial cells, indicating that SBP1 and SBP3 played no role in adherence in these assays. In contrast, both *sbp1* and *sbp3* knockout mutants showed significantly reduced invasion of respiratory epithelial cells compared to that of the wild type ($P < 0.005$ for both) (Fig. 2). Invasion of the mutants was approximately 40% of the wild-type level. We conclude that SBP1 and SBP3 play a role in invasion of respiratory epithelial cells by *M. catarrhalis*.

DISCUSSION

SBP2 of the ABC transporter system that is the focus of the current study was previously identified as a promising vaccine antigen for *M. catarrhalis* (25). SBP2 is highly conserved among strains and expresses epitopes on the bacterial surface, and these epitopes bind potentially protective antibodies. Furthermore, immunization with recombinant purified SBP2 induces enhanced clearance in the mouse pulmonary clearance model (25). In view of the potential importance of SBP2 as a vaccine candidate, the present study was undertaken to further characterize the SBPs encoded by the ABC transporter gene cluster that encodes SBP2 to better understand their function for the bacterium and to begin to understand their role in pathogenesis. Understanding the function of the vaccine antigen, SBP2, will contribute to vaccine development, for example, by guiding the development of functional assays to assess potentially protective responses.

The ABC transporter gene cluster encodes three SBPs that are homologous with amino acid transport substrate binding proteins of other Gram-negative bacteria in the range of 50% identity and 70% similarity (25). The current study shows the following: (i) that the SBPs in this gene cluster have binding specificity for individual basic amino acids, i.e., lysine (SBP1), arginine (SBP2), and ornithine (SBP3); (ii) that the SBPs function in the uptake of basic amino acids to support growth of *M. catarrhalis*; and (iii) that

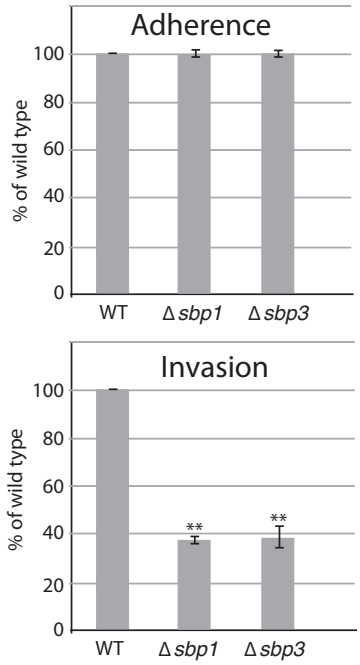

FIG 2 Results of adherence and invasion assays with the A549 respiratory epithelial cell line. Adherence and invasion are shown as a percentage of the wild-type values. Error bars show standard deviations of three independent experiments. **, $P < 0.005$ compared to results for the wild type (two-tailed *t* test).

SBP1 and -3 are required for optimal intracellular survival in human respiratory epithelial cells. One might speculate that SBP1 and SBP3 facilitate intracellular survival because lysine and ornithine may be limited in the intracellular environment. The observation that a mutant that lacks all three SBPs does not grow in chemically defined medium emphasizes the important function of this transporter in survival of *M. catarrhalis* in nutritionally depleted environments, including the middle ear, the lower airways, and intracellular environments, all sites where *M. catarrhalis* causes disease. Collectively, these observations are consistent with the concept that the SBPs in this ABC transporter are nutritional virulence factors that contribute to the capacity of *M. catarrhalis* to survive in the ecological niche of the human respiratory tract.

M. catarrhalis has a strict growth requirement for arginine (26, 36); thus, this gene cluster, and particularly SBP2 by mediating uptake of arginine, may be especially critical for the bacterium to colonize and infect the human respiratory tract. Most arginine that is present in the human respiratory tract is methylated because it is derived from proteolysis of host proteins (37, 38). The ratio of methylated arginine and L-arginine in the airways is called the "arginine paradox" whereby L-arginine is limited due to intracellular sequestration, competition for arginase, and production of nitric oxide (39, 40). *M. catarrhalis* requires L-arginine for growth and cannot use methylated arginine, indicating that specific arginine uptake systems are critical for survival in the respiratory tract (36). *M. catarrhalis* also has an oligopeptide permease transporter system that mediates uptake of arginine-containing peptides (36).

M. catarrhalis invades and survives inside host cells in the human respiratory tract. Heiniger et al. (41) demonstrated that cells in the tonsils and adenoids serve as a reservoir for *M. catarrhalis* to persist in the human respiratory tract. The observation that SBP1 and SBP3 partially mediate invasion of respiratory epithelial cells suggests that these proteins contribute to persistence of the bacterium. Although we were unable to assess invasion of cells specifically by mutants that did not express SBP2, one might speculate that SBP2 plays a nutritional role in intracellular persistence, given the importance of SBP2 in the viability of the bacterium, particularly in environments where arginine is limited.

The role of SBP2 in uptake of arginine may have important implications for SBP2 as a vaccine antigen. Immunization with SBP2 induces antibodies to surface epitopes; such antibodies may mediate protective responses through complement-mediated bactericidal killing, opsonization for phagocytosis, or the blocking of adherence and invasion. In addition, binding of antibodies to a functionally important surface molecule may inhibit the function of that molecule. In the case of SBP2, blocking the uptake of arginine could be lethal for *M. catarrhalis*. This scenario is analogous to the observation that inhibition of the glycerophosphodiesterase activity of protein D of *H. influenzae* is an important element in the protective responses to *H. influenzae* induced by the pneumococcal conjugate vaccine that has protein D as the carrier protein (42, 43).

The binding specificity of the SBPs indicates that this gene cluster is a lysine-arginine-ornithine (LAO) ABC transporter. The LAO transporters in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Pseudomonas aeruginosa* each have single substrate binding proteins that bind lysine, arginine, and ornithine (44–48). In contrast, this LAO transporter in *M. catarrhalis* has three separate SBPs encoded by individual genes. Each SBP has distinct binding affinities (Tables 1 and 2).

Berntsson et al. (49) proposed a classification system for SBPs that is based on structural alignment of publicly available crystal structures of SBPs. LAO SBPs belong to subcluster F-IV, which is a large subgroup of amino acid-binding proteins (49). SBPs in this subcluster often bind other amino acids in addition to their primary substrates, but generally with an affinity an order of magnitude lower. SBP1 and SBP3 of the LAO transporter of *M. catarrhalis* show this behavior in the experiments assessing their affinities for uptake of amino acids as nutrients (Table 2). In the thermal shift assay, SBP1, -2, and -3 show exquisite binding specificities to their primary substrates (Table 1), whereas growth experiments with mutants that express each of the SBPs individually detected binding to additional amino acids. The different assay systems used to detect binding may account for this difference. Thermal shift assays are performed with purified recombinant proteins, resulting in partial denaturing of the proteins, which affects their binding characteristics. During bacterial growth the proteins are in their native states. Furthermore, growth rate appears to be a more sensitive endpoint than thermal shift, allowing the detection of lower-affinity binding to secondary amino acid substrates.

This work advances our understanding of how *M. catarrhalis* takes up arginine and other basic amino acids and the important role that this uptake system plays in growth. The capacity of *M. catarrhalis* to cause infection in protected niches in the human respiratory tract, including the middle ear and the lower airways, depends on the bacterium's ability to survive and multiply in these hostile environments. The critical role of SBP2 in the uptake of arginine, for which *M. catarrhalis* has a strict growth requirement, may contribute to its potential as a vaccine candidate. Future investigations should be directed at understanding the effect of im-

mune responses on the function of the substrate binding proteins in this ABC transporter and how this may contribute to protective responses.

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