Peptide methionine sulfoxide reductase contributes to the maintenance of adhesins in three major pathogens

(type IV pili/type I fimbriae/*Neisseria gonorrhoeae*/*Streptococcus pneumoniae*/pilB)

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ABSTRACT Pathogenic bacteria rely on adhesins to bind to host tissues. Therefore, the maintenance of the functional properties of these extracellular macromolecules is essential for the pathogenicity of these microorganisms. We report that peptide methionine sulfoxide reductase (MsrA), a repair enzyme, contributes to the maintenance of adhesins in *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, and *Escherichia coli*. A screen of a library of pneumococcal mutants for loss of adherence uncovered a MsrA mutant with 75% reduced binding to GalNAc β 1–4Gal containing eukaryotic cell receptors that are present on type II lung cells and vascular endothelial cells. Subsequently, it was shown that an *E. coli* msrA mutant displayed decreased type I fimbriae-mediated, mannose-dependent, agglutination of erythrocytes. Previous work [Taha, M. K., So, M., Seifert, H. S., Billyard, E. & Marchal, C. (1988) EMBO J. 7, 4367–4378] has shown that mutants with defects in the *pilA*–*pilB* locus from *N. gonorrhoeae* were altered in their production of type IV pili. We show that pneumococcal MsrA and gonococcal PilB expressed in *E. coli* have MsrA activity. Together these data suggest that MsrA is required for the proper expression or maintenance of functional adhesins on the surfaces of these three major pathogenic bacteria.

Cellular recognition and attachment of pathogenic microorganisms to eukaryotic cells mediated by surface exposed macromolecules are critical steps in the successful colonization and subsequent invasion of tissues in the human host (1). For example, the majority of *Escherichia coli* strains express rigid filamentous appendages, type I fimbriae, that mediate adherence to mannose containing glycoconjugates on the surfaces of eukaryotic cells (2, 3). As another example, type IV pili and PII outer membrane proteins expressed on the surface of *Neisseria gonorrhoeae* mediate adherence, in a complex manner, to several classes of glycoconjugate-containing receptors on the surfaces of epithelial cells of the urogenital tract (1, 4). Work in one of our laboratories (H.R.M.) has focused on the interaction between adhesins expressed on the surface of *Streptococcus pneumoniae* and the ligands on type II lung cells (LC) and vascular endothelial cells (EC) (5–8). *S. pneumoniae* recognizes three classes of glycoconjugate-containing receptors on the surfaces of LC and EC. Pneumococci bind to glycoconjugate receptors containing the disaccharides GalNAc β 1–3Gal or GalNAc β 1–4Gal. A third receptor containing GlcNAc is expressed after these cells are treated with several pro-inflammatory agents and it is likely that this is the platelet-activating factor (PAF) receptor (9).

To define the nature of the ligands on the surface of pneumococcus responsible for binding to eukaryotic cells, we screened a library of mutants with defects in exported proteins

for the inability to bind to the various eukaryotic receptors. One mutant showed decreased binding to the GalNAc β 1–4Gal class of receptors. The gene disrupted in this mutant was shown by sequence similarity to encode a protein with homologs in several bacteria including *E. coli* (MsrA), *N. gonorrhoeae* (PilB), *Haemophilus influenzae* (10), and *Mycoplasma genitalium* (11) and the eukaryotic organism *Caenorhabditis elegans* (12). The characteristics of MsrA, a protein that reduces methionine sulfoxide residues in proteins, have been extensively studied (13–16). In the present study, it is demonstrated that PilB, which is implicated in pili production, and the pneumococcal protein have MsrA activity. In addition, an *E. coli* msrA mutant also exhibits a reduced mannose-dependent ability to bind to receptors on eukaryotic cells. Based on these observations, we propose that MsrA is critical for the production and/or the maintenance of the functional properties of adhesins in the three major pathogens *E. coli*, *N. gonorrhoeae*, and *S. pneumoniae*.

MATERIALS AND METHODS

Strains, Bacterial Growth, and Molecular Genetics. The pneumococcal strain used in this study was R6x, which is a derivative of the noncapsulated Rockefeller University strain R36A (17). *S. pneumoniae* were routinely plated on tryptic soy agar (Difco) supplemented with sheep blood (Micropure Medical, White Bear Lake, MN) to a final concentration of 3% (vol/vol). Liquid cultures of pneumococcus were grown in a semisynthetic casein hydrolysate medium supplemented with a yeast extract (18). For the selection and maintenance of pneumococci containing chromosomally integrated plasmids, bacteria were grown in the presence of erythromycin at 0.5 μ g/ml.

Established protocols were used for the genetic transformation of pneumococci as well as the recovery of spontaneously excised plasmids (19–21). Mutant libraries of pneumococci containing the nonreplicating chromosomally integrated plasmids pHRM100 and pHRM104 were created as previously described (19).

The *E. coli* strains used in these studies were MC 1061 and SK 8779 (*msrA*1::kan). The latter is a mutant strain of MC1061 carrying a disrupted *msrA* gene (22).

For hemagglutination studies, *E. coli* strains were grown in Luria broth (LB) under static conditions for 48–72 h. To maintain plasmids in mutant strains, ampicillin and/or kanamycin (50 μ g/ml) was added to the culture medium.

The *N. gonorrhoeae* strains used were derivatives of MS11A (P⁺Tr) (23) and were grown on GCB agar (Difco) with supplements (24). Isogenic nonpiliated derivatives of the wild-

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Abbreviations: LC, type II lung epithelial cells; EC, human vein endothelial cells; IPTG, isopropyl β -D-thiogalactopyranoside; MsrA, peptide methionine sulfoxide reductase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U41735).

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type and *pilB* mutants were constructed by replacement of *pilE* coding sequences with antibiotic resistance cassettes using described techniques (25). All strains were determined to be opa⁻ (Tr) by colony morphology as well as by immunoblot analysis with anti-Opa sera.

Bacterial Adherence. The assays for the adherence of pneumococci to LC, EC, and immobilized glycoconjugates were performed as previously described (6). The glycoconjugates used to detect binding specificities for the disaccharides GalNAc β 1-4Gal (asialo-GM1 and asialo-GM2), GalNAc β 1-3Gal (globoside), or the platelet-activating factor receptor (α 1-acid glycoprotein) were purchased from Sigma.

Gonococci were assayed for adherence to A431 epithelial cells (ATCC CRL 1555). Cells were cultured in DMEM (GIBCO) supplemented with 10% heat-inactivated calf serum at 37°C in a humidified 5% CO₂ atmosphere. Bacteria harvested from GCB agar plates after 18–20 h growth were swabbed into GC broth and diluted to the appropriate density in DMEM immediately before infection. Cells grown in 24-well trays to near confluence were infected with gonococci (multiplicity of infection = 10). After 7 h incubation, nonadherent bacteria were removed by washing 5 $\times$ with sterile PBS. Total bacterial counts were determined by removing the supernatant, lifting the cells with PBS and 0.5 mM EDTA, and plating dilutions of this mixture onto GCB plates.

Hemagglutination. Agglutination of guinea pig erythrocytes was used as an assay for type I fimbriae-mediated, mannose-dependent adherence to eukaryotic cell receptors. Serial dilutions of bacterial suspensions of *E. coli* were mixed with an equal volume of 2% guinea pig erythrocytes (Micropure Medical). The titer of hemagglutination was determined as the dilution exhibiting no visible agglutination after 2 h incubation at 4°C. In some assays, α -methyl-D-mannoside was added to inhibit hemagglutination (26, 27).

DNA Manipulations. Most procedures were performed according to standard methods (28). The recovery in *E. coli* of integrated plasmids harboring pneumococcal DNA from mutant strains of pneumococcus has been described elsewhere (7, 19, 20).

The genetic locus containing the pneumococcal *msrA* gene, as well as adjacent DNA, was obtained by inverse PCR. A plasmid (pTW1) harboring a 600-bp sequence of pneumococcal DNA from the adhesin defective mutant SPRU40 was established in *E. coli* and the entire sequence of this insert was determined. Outward facing primers were designed to the ends of this DNA. Chromosomal DNA from the parent strain, R6x, was digested with *EcoRI*, self-ligated, and then amplified by the PCR using the primers to the insert from pTW1. Conditions for the PCR were 94°C for 1 min for denaturing, 55°C for 1 min for annealing, and 72°C for 2 min for extension, for 25 cycles. A single 3.5-kb product was obtained and sequenced with primers specific to this amplified product. DNA sequence was determined by the *Taq* dye terminator cycle sequencing method on an automated DNA sequencer (model 373, Applied Biosystems) operated by the Protein/DNA Technology Center at Rockefeller University. Derived protein sequences were analyzed for similarity to sequences deposited in the database at the National Center for Biotechnology Information (Washington, DC) using the BLAST algorithm (29). Alignments were performed with the PILEUP program [available on version 7 of the Genetics Computer Group (Madison, WI) sequence analysis software package] (30).

Enzyme Activity. MsrA activity was determined as described (31) using 150 pmol of *N*-acetyl-[³H]methionine sulfoxide as substrate. Cell free extracts of *S. pneumoniae* were prepared by sonication of a suspension of mid-log phase bacteria in the presence of glass beads in PBS containing 2 mM phenylmethylsulfonyl fluoride. The extract was centrifuged at 32,000 $\times$ g for 20 min and the supernatant assayed for enzymatic activity. *E. coli* cell free extracts were obtained by sonication of a

cellular suspension as described previously (22). After centrifugation, the supernatants were analyzed for enzyme activity.

MsrA and PilB Expression in *E. coli*. The gene for the pneumococcal *msrA* was synthesized by the PCR using a 5' sense primer containing an *EcoRI* site (5'-ATAAAGATGGAATTCATTTATCTAGCAGGTTGG) and a 3' reverse complement primer with an added *XbaI* restriction site (5'-CCCTTCTATTCATATTCTAGAAATGAAG). The *pilB* gene was also made by the PCR using a 5' sense primer containing an *EcoRI* site (5'-CATCAAAATGGAATTCCTACTTTCTTTTCCC) and a 3' reverse complement primer with an added *XbaI* site (5'-CCGGGTTAGGTCTAGATGCGGCTTATTTTCAC). The PCR was performed for 30 cycles of 30 sec at 94°C, 60 sec at 50°C, and 90 sec at 72°C, using chromosomal *S. pneumoniae* DNA prepared in our laboratory or *N. gonorrhoeae* DNA kindly provided by Emil Gotschlich (Rockefeller University). The amplified products were each inserted into the expression plasmid pTrc99A (Pharmacia). The pTrc99A vectors harboring the pneumococcal *msrA* and *pilB* genes were denoted as pJM300, and pJM400, respectively. *E. coli* mutant cells lacking the *msrA* gene (22) were transformed with an aliquot of each ligation mixture, and grown in LB containing ampicillin and kanamycin (50 μ g/ml each) to an A₆₀₀ of 0.4. Isopropyl β -D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM and the bacteria were cultured for an additional 4 h. Bacteria were collected by centrifugation, suspended in a buffer containing 25 mM Tris-HCl, pH 7.4, and disrupted by sonication. The lysates were centrifuged and the supernatants were analyzed for MsrA activity (31). A gene fusion between pneumococcal *msrA* and glutathione transferase was constructed using pGEX-2T (Pharmacia).

Statistics. Data were analyzed using Student's *t* test. Results were considered statistically significant at *P* < 0.05. Data for the pneumococcal adherence assays is presented as the mean of six individual measurements; the standard deviation of any value did not vary by greater than 15% of that value.

RESULTS

Identification of a Pneumococcal Adherence Deficient Mutant. Initially, a bank of mutants with defects in exported proteins was generated by insertion duplication mutagenesis with two *phoA* (alkaline phosphatase) fusion vectors described elsewhere (19). Mutants positive for alkaline phosphatase activity (*n* = 150) were screened for loss of adherence to eukaryotic cells or to glycoconjugates that were determined to best represent the eukaryotic cellular receptors for pneumococcus (6, 32, 33). One mutant, SPRU40, showed reduced adherence to LC and EC (Fig. 1). To confirm that the loss of adherence was the result of the marked mutation, the allele from SPRU40 was recovered and transferred by gene replacement to the parent strain and a similar loss of adherence was observed (data not shown). Residual binding of SPRU40 to LC was inhibited by an exogenous glycoconjugate containing the disaccharide GalNAc β 1-3Gal, whereas a glycoconjugate containing GalNAc β 1-4Gal had no significant effect on residual binding (Fig. 2). A similar pattern of glycoconjugate-mediated inhibition was observed in adherence assays between pneumococci and EC (data not shown). In further studies, the ability to adhere directly to immobilized glycoconjugate analogs was assessed. The binding of SPRU40 to glycoconjugates containing GalNAc β 1-4Gal was reduced by 75% compared with the parent strain, whereas binding to other glycoconjugates was unchanged (Table 1). These data suggest that the locus disrupted in SPRU40 is required for the production of a functional ligand that binds to the GalNAc β 1-4Gal receptor present on eukaryotic cells.

Sequence Analysis of the Locus Disrupted in SPRU40. Recovery and analysis of the altered locus in SPRU40 showed a gene consisting of 936 bases that would encode a protein of

FIG. 1. Adherence of R6x (○) and SPRU40 (●) to LC (A) and EC (B). Adherence was determined microscopically as the number of attached fluorescein-labeled bacteria per 100 eukaryotic cells. Data are the mean $\pm$ SD of six experiments. Adherence of SPRU40 to both LC and EC was significantly reduced ($P < 0.05$) at 10^7 to 10^9 cfu/ml, compared with the parent strain R6x.

312 amino acids with a predicted molecular mass of 35 kDa (Fig. 3). A potential ribosome binding site was found 15 bases

FIG. 2. Effect of glycoconjugates on adherence of pneumococci to LC. SPRU40 were incubated with asialo-GM1, a GalNAcβ1-4Gal containing glycoprotein, or globoside, a GalNAcβ1-3Gal containing glycoprotein for 15 min before the addition of the bacteria to the LC monolayer. Adherence was determined microscopically as the number of attached fluorescein-labeled bacteria per 100 LC. Data are the mean $\pm$ SD of six experiments. Adherence of SPRU40 to LC was significantly reduced ($P < 0.05$) by preincubation with 100 and 500 μ M GalNAcβ1-3 Gal, compared with control (no sugar).

Table 1. Direct adherence of pneumococci to immobilized glycoconjugates

Strain	Adherent bacteria/0.0625 mm ²			Platelet-activating factor receptor
	No sugar	GalNAcβ1-4Gal	GalNAcβ1-3Gal	
R6x	27 $\pm$ 7	398 $\pm$ 63	161 $\pm$ 16	162 $\pm$ 17
SPRU40	32 $\pm$ 10	103 $\pm$ 15*	158 $\pm$ 15	165 $\pm$ 21

Pneumococci (10^7 colony-forming units per ml) were incubated with immobilized GalNAcβ1-4Gal (asialo-GM2, 100 μ M), GalNAcβ1-3Gal (globoside, 100 μ M), or platelet-activating factor receptor (α 1-acid glycoprotein, 100 μ M) for 30 min at 37°C. Data are the mean $\pm$ SD of six experiments.

*Significantly less ($P < 0.05$) than adherence by the parent strain, R6x.

upstream from the ATG initiation codon and a potential transcription terminator 4 bases downstream from the TAA stop codon. Comparison of the derived amino acid sequence to entries in the current DNA sequence libraries revealed several homologs. The deduced amino acid sequence of the pneumococcal gene displayed >40% identity with MsrA from *E. coli* (34) and 50% identity with the carboxyl terminal region of PilB from *N. gonorrhoeae*, a putative transcriptional regulator (35). Within the amino terminal region of PilB (Fig. 3B, positions 68–71), there is a domain that contains a close match to the consensus sequence CysGlyProCys, which is the active site for protein oxidoreductases found in thioredoxin and thioredoxin-like proteins. This region is most like the domains found in a putative disulfide exchange protein (DsbE) from *H. influenzae* (10) and thioredoxin and thioredoxin like proteins from *Rhodobacter sphaeroides* (36, 37). None of these homologs suggested that the pneumococcal gene encoded a structural element that serves as an adhesin. Since, as shown below, we demonstrate that MsrA activity is associated with the protein product of this gene, it is designated *msrA* after its *E. coli* counterpart.

Upstream of the pneumococcal *msrA*, in the opposite orientation, are four conserved DNA sequences with >90% identity to the consensus sequences for several pneumococcal Box elements (38). These sequences have the potential to form stem-loop structures and it has been proposed that they function as regulatory elements. Upstream from the start codon (29 bp) of *msrA* is one copy of box A followed by two copies of box B and one copy of box C.

Adjacent to *msrA* are two genetic elements that potentially express gene products with homologs in other bacteria (Fig. 3A). Upstream of the box region is a homolog of the homoserine kinase (*thrB*) from *Bacillus subtilis* (39). In this organism, this enzyme is the fourth component of five enzymes required for threonine biosynthesis. The biosynthetic pathways for lysine, methionine, and isoleucine diverge from the threonine biosynthetic pathway. Downstream of *msrA* is a member of the ABC family of transporters required for the active transport of many diverse molecules across lipid membranes (for review, see ref. 40). This homolog is most like HlyB found in many bacteria, which is dedicated to the secretion of hemolysins across bacterial membranes. We note that the hallmark ATP binding motif or "Walker" box found in all of these homologs is also present in the deduced amino acid sequence of the pneumococcal gene (41).

Peptide Methionine Sulfoxide Reductase Activity Associated with MsrA and PilB. Experimental data showed that the *E. coli* MsrA, a homolog of the pneumococcal MsrA, is an enzyme that reduces methionine sulfoxide residues to methionine (34, 42) whereas other data suggested that PilB, the homolog from *N. gonorrhoeae*, is a sensor component of the two component family of sensor regulators (35, 43). We undertook a series of experiments to resolve this incongruity.

FIG. 3. Structural organization and sequence analysis of the pneumococcal *msrA*. (A) Structural organization of the pneumococcal *msrA* and adjacent DNA. (B) Amino acid sequence alignment of the pneumococcal MsrA (pMsrA) with PilB and the *E. coli* MsrA. A conserved domain corresponding to the active site of protein oxidoreductases present in PilB, DsbE, a disulfide exchange protein from *H. influenzae* (10), and TrxA from *R. sphaeroides* (36) is also indicated (positions 68–71). Amino acid sequence alignment was performed with the PILEUP program (available on version 7 of the Genetics Computer Group sequence analysis software package) (30). Identical or conserved amino acids are indicated (uppercase). A consensus sequence was derived on the basis of a comparison of each individual sequence associated with pneumococcal *msrA* and alignment of the deduced amino acid sequences of the homologs of the *S. pneumoniae*, *E. coli*, and *N. gonorrhoeae* MsrA.

First, compared with extracts of the parent pneumococcal strain (which had a MsrA specific activity of 0.34 pmol/h/μg protein), there was no detectable MsrA activity associated with the pneumococcal strain SPRU40 (which harbors a disrupted *msrA*). This result, in addition to the sequence similarity between the two genes, suggested that the pneumococcal *msrA* encodes a MsrA. Second, the pneumococcal *msrA* and *pilB* genes were expressed in *E. coli* under the control of an

inducible promoter harbored on appropriate expression vectors, and these strains were assessed for MsrA activity. The host strain for these vectors was an *msrA* mutant that has no MsrA enzyme activity (22). These cells were then grown in the presence or absence of the inducer IPTG. Fig. 4 shows that only in the presence of IPTG were two proteins of ≈35 kDa and 32 kDa synthesized (lane 2 vs. 5 and lane 3 vs. 6) in cells transformed with the plasmids bearing the pneumococcal *msrA* and *pilB*, respectively. The molecular weight of the expressed pneumococcal protein is in good agreement with its predicted molecular weight from the deduced amino acid sequence. The induced protein from the strain harboring *pilB* is smaller than its predicted molecular mass of 58 kDa (35). It is possible that this is a proteolytic product of PilB.

To determine whether the expression of these proteins correlated with MsrA activity, the cells harboring the inducible pneumococcal *msrA* and *pilB* genes were assayed for this activity. Table 2 shows that in the absence of IPTG there was no enzyme activity in any of the transformants. After the addition of IPTG only strains harboring the inducible *msrA* (pJM300) and *pilB* (pJM400) genes produced MsrA activity. Finally, a gene fusion between pneumococcal *msrA* and glutathione transferase was constructed, the protein product purified and shown to have MsrA activity (11.1 pmol/h/μg protein). Therefore, we conclude that pneumococcal MsrA and gonococcal PilB are homologs of *E. coli* MsrA and that all three possess MsrA enzyme activity.

Peptide Methionine Sulfoxide Reductase Affects *E. coli*-Mediated Hemagglutination. Mutants with defects in the *pilA*–*pilB* locus are altered in levels of pilin expression, whereas pneumococcal mutants with defects in *msrA* have alterations in surface ligands responsible for binding to eukaryotic cell receptors. Therefore, we tested MsrA defective *E. coli* strains for changes in binding to eukaryotic cell receptors using a model system for the ability of *E. coli* that express type I fimbriae to agglutinate erythrocytes in a mannose-dependent manner. An eight-fold decrease in hemagglutination was observed for an *msrA* defective *E. coli* strain. Hemagglutination of the mutant was restored to the levels observed in the parent strain with the introduction of a plasmid containing *msrA* expressed from its own promoter (34). The activity restored by the plasmid correlated with α-methyl-D-mannoside inhibitable hemagglutination, consistent with type I fimbriae (Table 3).

Peptide Methionine Sulfoxide Reductase Affects Adherence of *N. gonorrhoeae* to Eukaryotic Cells. It was previously shown that a *pilB* mutant of *N. gonorrhoeae* was hyperpiliated (35),

FIG. 4. Expression of MsrA and PilB. The genes for PilB and pneumococcal MsrA were cloned into the pTrc99A expression vector just down stream of an IPTG inducible promoter and the corresponding plasmids transformed into SK8779 (*msrA*1::kan). These strains were then grown with or without the addition of the inducer. Lanes 1 and 4; cells transformed with pTrc99A; lanes 2 and 5, cells transformed with pJM300 (*msrA*); lanes 3 and 6, cells transformed with pJM400 (*pilB*).

Table 2. Expression of pneumococcal MsrA and PilB in an *E. coli* MsrA mutant

Plasmid	MsrA specific activity (pmol <i>N</i> -acetyl met/h/ μ g)	
	–IPTG	+IPTG
pTrc99A	0.00	0.00
pJM300	0.00	4.44
pJM400	0.00	6.50

The construction of the plasmids, expression of the *msrA* and *pilB* genes, and the MsrA enzyme assay are as described.

which might suggest a hyperadherent phenotype. In adherence assays at a multiplicity of infection of 10, 88% of *pilB* mutant gonococci adhered to A431 epithelial cells compared with 34% of wild-type gonococci. In the same assays, adherence of nonpilated derivatives of the wild-type and *pilB* mutant strains were reduced to 0.24% and 0.23%, respectively. This demonstrates that hyperpilated *pilB* mutant gonococci are also hyperadherent, but the *pilB* mutation has no effect on adhesion in a nonpilated background.

DISCUSSION

The functional properties of the molecules on the surfaces of microbial pathogens and host cells are essential to defining the molecular characteristics of an infectious disease. Many elements are required for the expression, assembly, positioning and maintenance of these surface molecules. In our efforts to characterize the adherence properties of *S. pneumoniae* to eukaryotic cells (5, 7, 9, 44, 45), we have defined several pneumococcal elements that contribute to the process of adherence (5, 7). To date, no specific surface proteins have been shown to function as adhesins in pneumococcus although several membrane associated molecules contribute to the expression of adhesins (5, 7). Using a strategy that couples bacterial mutagenesis to an *in vitro* adherence assay, we identified an adhesion deficient mutant with a defect in a gene, designated *msrA*, that codes for an enzyme, peptide methionine sulfoxide reductase, that may function to maintain the functional properties of a pneumococcal adhesin. It has been shown that *S. pneumoniae* recognizes several specific glycoconjugates on the surfaces of LC and EC (6, 32). The pneumococcal *msrA* defective mutant displayed substantially decreased adherence to LC and EC as a result of a lack of recognition of the specific GalNAc β 1–4Gal ligand.

The oxidation state of sulfur-containing amino acids affects the functional properties of many proteins, and both prokaryotic and eukaryotic organisms have enzymes that alter the redox state of these amino acids. Thioredoxin, glutaredoxin, and protein disulfide isomerases are examples of intracellular proteins that have been shown to be required for the maintenance of functional properties of a wide variety of proteins (for review, see ref. 46). A growing family of dedicated protein disulfide oxidoreductases have been characterized in a variety

of bacteria. One class of enzymes catalyzes the oxidation of cysteine residues promoting correct inter- and intramolecular disulfide bond formation, thus conveying functionality to these proteins (refs. 47–50; for review, see ref. 51). It has been shown that these enzymes are required for the correct folding and enzymatic properties of the exported protein, alkaline phosphatase, from *E. coli* (49, 50) and the secretion of a variety of virulence determinants in *Vibrio cholerae*, including a colonization factor and presumed adhesin (52). An example of one of these enzymes has also been shown to be required for the process of natural transformation in *H. influenzae* (53).

A second class of enzyme, MsrA, catalyzes the reversible oxidation–reduction of methionine sulfoxide in proteins to methionine (15). The pneumococcal *msrA* gene product expressed in *E. coli* has MsrA activity. The oxidation of methionine in proteins has been shown to result in loss of biological activity, which is restored by MsrA (15). This seems to be the most likely molecular mechanism responsible for affecting the production of the adhesins associated with the bacteria presented in the current study. Methionine in proteins may be oxidized by biologically reactive oxygen intermediates such as hydrogen peroxide and superoxide anion. Pathogenic bacteria are challenged by these molecules as the products of normal bacterial metabolism (54, 55) or as toxins produced by the host's immune defense cells (56, 57). It is of note that pneumococcus is a catalase-deficient organism and is particularly susceptible to reactive oxygen intermediates.

The results of a search of the most recent genetic and protein databases revealed homologs of the pneumococcal MsrA in *E. coli*, *N. gonorrhoeae* (PilB), *H. influenzae* (10), *M. genitalium* (11), and *C. elegans* (12). To explore a link between this enzyme and other bacterial adhesins, the adherence properties of a type I fimbriated *E. coli* *msrA* defective mutant was analyzed. The absence of functional MsrA in type I fimbriated *E. coli* resulted in decreased mannose-dependent agglutination of erythrocytes. This suggests that MsrA is required for the production or maintenance of surface fimbriae on this pathogen. Electron microscopy of the *E. coli* strains revealed no apparent phenotypic differences in the fimbriae of the *msrA* defective mutants and parent strains (data not shown). FimH, present at the tip of the fimbriae, has been implicated as the specific molecule responsible for this type of adherence (2). Immunogold labeling of the mutant and the parent strain revealed no apparent differences in quantity or distribution of FimH (data not shown). Therefore, the effect of MsrA on the maintenance of this adhesin was not revealed by this type of analysis.

PilB is the best characterized element associated with the production of an adhesin associated with the bacteria considered in this study. Originally discovered as part of a regulatory element (*pilA-pilB*), it was shown that the gene products of this locus control transcription of *pilE*. *PilE* is the expression locus for pilin, the major protein subunit of gonococcal pili (35, 43). It has been proposed that PilA–PilB are similar to the two component family of sensor regulators (43, 58, 59). Experimental data support the notion that PilA is the response regulator of this system and controls pilin expression at the level of transcription in a manner that may be dependent on the novel GTPase activity associated with this protein (60, 61). The model proposes that PilB functions as the sensor kinase of this system that phosphorylates PilA to alter its ability to affect pilin transcription (43, 58, 59).

In this study we show that recombinant PilB has MsrA activity and that the N-terminal region contains a domain highly similar to the active site (CysGlyProCys) associated with thioredoxin-like proteins. It is of note that this is the first example of a single protein that contains both thioredoxin-like and reductase domains. If, as originally proposed, PilB acts in concert with PilA to regulate the expression of *pilE*, it may not be by the traditional phospho-relay system common to two component sensor regu-

Table 3. Effect of MsrA on hemagglutination by *E. coli*

Strain	MsrA enzyme activity	Hemaggluti- nation activity*	Conc. (μ g/ml) α -Me-D-mannoside for inhibition†
Parent MC1061	+	1:16	250
SK8779	–	1:2	30
SK8779 transformed with plasmid carrying <i>msrA</i>	+	1:16	125

Data presented are a representative experiment of five similar experiments.

*Dilution of *E. coli* at which visible hemagglutination was lost.

†Minimal concentration required for complete inhibition.

lators. Recent data from *in vitro* studies showed that PilB was not phosphorylated and there was no exchange of phosphate with PilA (59). Therefore, we propose that PilB may affect the transcription of *pilE* by the inherent oxido-reductase activity of the protein by affecting the transcriptional activator function of PilA. This is not without precedent. In the face of strong oxidants, a series of stress response elements are up regulated by the transcription factor NF- κ B in eukaryotic cells (62) and it has been shown that thioredoxin directly affects NF- κ B mediated gene transcription by reduction of a disulfide bond in this transcription factor (63, 64).

Mutants of *N. gonorrhoeae* with defects in *pilB* have been shown (35) to be hyperpilated, as well as hyperadherent, which is in contrast to the *E. coli* and pneumococcal mutants with defects in MsrA homologs that are adhesion deficient. Because the targets for the MsrA activities in these bacteria have not been identified, we can only speculate on a mechanism of action. It is possible that MsrA homologs may be affecting gene transcription in the case of *N. gonorrhoeae*, whereas in the other bacteria the mechanism may be at other levels of gene regulation or as a chaperone, maintaining a functional adhesion.

In summary, we have shown that a specific class of enzymes, MsrA, is responsible for the production of functional adhesins on the surfaces of three major bacterial pathogens. From this and other studies, it is clear that the oxidative state of the sulfur groups of both methionine and cysteine within proteins contributes to the expression and maintenance of the functional properties of the molecules on the surface of bacteria. Finally, because MsrA serves this critical function for three major pathogens of both Gram-negative and Gram-positive types, we suggest that it may be a useful target for anti-infective therapy.

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