Characterization of the capsule-binding protein of *Mycoplasma dispar*

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SUMMARY

Mycoplasma dispar is a capsulated mycoplasma that can cause chronic pneumonia in calves. The capsule of *M. dispar* exerts inhibitory effects on certain functions of bovine alveolar macrophages, specifically the production of tumor necrosis factor and interleukin 1, and glucose consumption. A 126 kDa protein, which we call capsule binding protein (CBP), has been observed to bind specifically to purified capsules of *M. dispar*. Polyclonal serum against capsule binding protein of *M. dispar* was made by immunizing rabbits with affinity purified CBP. Triton X-114 phase fractionation of mycoplasmas followed by analysis by SDS-PAGE and immunoblotting with the polyclonal serum identified that the CBP has hydrophobic fragments. Trypsin treatment of intact mycoplasma cells further demonstrated that CBP was a membrane-associated protein. Trypsin treatment of intact cells also generated a 66 kDa protein fragment that could bind to *M. dispar* capsules. Carboxypeptidase Y treatment of intact mycoplasma showed that CBP could be cleaved by this enzyme. This demonstrated that the C-terminal end of the CBP is on the outside of *M. dispar* cells. This protein may be similar to other C-terminal- exposed proteins of prokaryotes with binding properties, or may have other functions beyond its currently demonstrated binding properties such as the streptococcal glycosyltransferases which have binding and synthase activities in separate external domains.

INTRODUCTION

Mycoplasma dispar (*M. dispar*) was first isolated and characterized in England (48, 49). It has since been one of the most frequently isolated mycoplasmas from pneumonic lungs of calves in England (49), Australia (144), USA (12), Japan (79) and Denmark (99). This mycoplasma appears to be a common inhabitant of the respiratory tract of healthy and diseased calves (49, 161, 12, 99, 153). The pathogenicity of *M. dispar* has been established by experiments in gnotobiotic calves (64, 50), by inoculations into the bovine mammary gland (17), in vitro by experiments on tracheal organ cultures (160) and also by epidemiological evidence (120, 153). Experimental studies (152) have demonstrated that *M. dispar*, when allowed to infect young calves naturally, effectively and extensively colonized their respiratory tract. The colonization was not only restricted to the upper parts of the respiratory tract but also involved the tracheobronchial tree. *M. dispar* cells were restricted to the margin of the ciliated epithelium (162). There they caused alterations of the ciliary apparatus which include degeneration of the respiratory epithelial cells and loss of the ciliary activity. It was also demonstrated that *M. dispar* could impair lung clearance of other bacteria (5). *M. dispar* rarely causes mortality by itself, but it is very important as a primary or concurrent agent of pneumonic infection in cattle (164, 35).

Experiments (63) with *M. dispar* have shown that the immune response of young calves to *M. dispar* is defective and it is unlikely to be entirely due to a general immaturity of the immune system of the calves since the response to *M. bovis* was not reduced to the same extent. It was also demonstrated that this

mycoplasma species could induce immunosuppression when injected in combination with other antigens and exerted an inhibitory effect on the mixed lymphocyte response to mitogens (30). It was suggested that this suppressive effect could be related to the avidity with which this mycoplasma species adhered to the host cell membrane, resulting in alteration in the membrane/receptor structures on the host cell and the production of a suppressive factor.

M. dispar was shown to produce a capsule in vitro, which could be visualized by electron microscopy following reaction with ruthenium red (62). The capsule of *M. dispar* posseses no obvious structure and extends for 17 to 24 nm beyond the cytoplastic membrane. Further studies confirmed that *M. dispar* does produce capsule *in vivo*, i. e. in infected calves, and that such capsules share antigenic determinants with capsules produced by this mycoplasma under *in vitro* conditions (4). The chemical composition of the capsules is a polymer of galactose and galacturonic acid (9).

Recently, it was demonstrated that encapsulated *M. dispar* or even purified *M. dispar* capsule exerted inhibitory effects on certain functions of bovine alveolar macrophages, specifically the production of tumor necrosis factor and interleukin I, and reduced glucose consumption (6). It is assumed that the antiphagocytic activity of *M. dispar* may enable the organism to evade phagocytosis in vivo, leading to a tendency towards a chronic infection in calves.

A 126 kDa protein (we call this protein capsule-binding protein, CBP) of *M. dispar* that can be radio-labeled with ^{35}S-methionine was shown to be co-regulated with capsule production, increasing in abundance as capsule is expressed. This CBP was also shown to have capsule-binding properties, since it

could be captured from *M. dispar* lysates by beads covered with purified *M. dispar* capsule (Rosenbusch, R., personal communication). A common mechanism of capsule production among gram positive *Streptococcus* species is the use of glucosyltransferase enzymes. These proteins have polysaccharide-synthesis and binding properties (80, 93, 94, 186). Because mycoplasma and streptococci evolved from a common ancestor (185, 119, 180), they may share some similarity in capsule production and capsule binding. Therefore, we presume that the 126 kDa protein may be associated with capsule production and may well have both a capsule-binding and a catalytic domain.

The main objective of this research project is to characterize this 126 kDa protein to see if the protein is a membrane integrated protein and its C-terminus is external, and to locate its capsule binding domain.

LITERATURE REVIEW

This literature review includes three parts : a general review of mycoplasma, a general review of capsule, and a review focused on the advances in capsule binding protein (CBP).

Mycoplasmas

The term "mycoplasmas" has been used rather loosely to denote any species in the class *Mollicutes*, whereas the terms acholeplasmas, ureaplasmas, spiroplasmas, anaeroplasmas, and asteroleplasmas are used when reference is made to specific members in the corresponding genera. So the name Mollicutes is suggested to be a common name for any member of the class. Nevertheless, "mycoplasmas" is still widely used in its broader sense (146).

The mycoplasmas are the smallest self-replicating prokaryotes (110). The single most important characteristic that distinguishes the mycoplasmas from other prokaryotes is their total lack of a cell wall (145). These organisms can filter through 450 nm pore diameter filters and produce minute colonies with typical fried-egg appearance in the case of most species.

Mycoplasmas usually exhibit an extremely simple ultrastructure. The mycoplasma cell is bounded by a plasma membrane and the enclosed cytoplasm contains ribosomes and a circular double-stranded DNA molecule. And the minute mycoplasma cells contain only the minimum sets of organelles and metabolic pathways essential for cellular growth and replication. The

mycoplasma genome is typically prokaryotic (185). However, the size of the *Mycoplasma* and *Ureaplasma* genome (500 to 800 kbp) is the smallest recorded for any self-replicating prokaryote, being approximately one-sixth the size of the *Escherichia coli* genome and approximately one-half the size of the genome of *Rickettsia*, while the organisms in the other mycoplasma genera are considered to have genomes double this size (112, 113). The mycoplasmas have an extremely low G+C content of mycoplasmal DNA. With very few exceptions the G+C content of mycoplasma genomes is within the range of 24 to 30 mole% (185).

The basis for the current classification system was established by Edward and Freundt (27). Classification of an organism within the class *Mollicutes* depends primarily on absence of a cell wall, filterability through a membrane filter of 450 nm diameter and production of minute colonies with fried-egg appearance. Subdivision of orders within this class is based on nutritional and morphologic criteria. Molecular tools in taxonomy include genome size, genomic base composition and DNA modification, DNA-DNA hybridization, cleavage patterns and genomic maps and specific genomic sequences (34). The class *Mollicutes* has three orders. Order I is *Mycoplasmatales* which has two families. Family I is *Mycoplasmataceae,* containing two genera. They are *Mycoplasma* and *Ureaplasma*. Differentiation between these two established genera depends on the capability of members of *Ureaplasma* to hydrolyze urea, with the production of ammonia and CO_2 (128). Family II is *Spiroplasmataceae* with one genus, *Spiroplasm*a. Order II is *Acholeplasmatales*. It has one family, *Acholeplasmataceae*, and this family has one genus, *Acholeplasma*. Order III is

Anaeroplasmatales. It has one family, *Anaeroplasmataceae*, and this family has two genera, *Anaeroplasma* and *Asteroleplasma*. There are also some mycoplasma-like organisms that are not cultivable and remain unclassified.

Almost all of the mycoplasmas are parasites of man, animals, anthropods, and plants. Although some mycoplasmas are commensals and belong to the normal flora, most species are pathogens. Mycoplasmas are distributed widely throughout the animal kingdom. Despite their ubiquitousness, mycoplasmas usually exhibit a rather strict host and tissue specificity (111). The customary habitat of the organisms in animal hosts is mucous membrane surfaces; accordingly they may be found in the respiratory, gastrointestinal and urogenital tracts. They can also be found in serosal surfaces such as those of joints. Many of the diseases caused by the mycoplasmas are chronic, life-long and difficult to cure (178).

In the past three decades several models for mycoplasma evolution have been formulated. Neimark (101, 102, 103) proposed that mycoplasmas were polyphyletic and presumably had arisen by degenerate evolution. According to this model, the mycoplasma would have arisen after evolution and diversification of the bacteria and different species are not phylogenetically related; instead, they would originate from different branches of the bacterial phylogenetic trees. Another model was proposed by Morowitz and Wallace (96, 179). In this model, mycoplasma was thought to arise very early in the evolution of living forms and be the precursors of the bacteria. But based on the 5S and 16S rRNA sequence, Woese, Maniloff, and coworkers demonstrated that none of these two models was correct (89, 185). Therefore, they proposed another model (185). In this

model, the basic ideas are: (i) the mycoplasmas are monophyletic, (ii) the mycoplasmas are from a branch of the gram-positive bacteria with DNAs having low G+C contents, and (iii) the evolution rate of the mycoplasma is fast. This model was confirmed and improved by subsequent studies (119, 180, 85). The mycoplasma phylogenetic order is *Asteroleplasma*, *Anaeroplasma* and *Acholeplasma*, *Spiroplasma*, *Mycoplasma,* and *Ureaplasma.* Although the mycoplasma phylogenetic tree is monophyletic, species currently included in the genus *Mycoplasma* are polyphyletic, having originated as three independent branches from the *Spiroplasma* branch (88). Mycoplasmas are evolving about 50% more rapidly than the *Lactobacillus* group, from which the mycoplasmas arose (184). The *Lactobacillus* group contains *Lactobacillus*, *Bacillus*, and *Streptococcus* species and two unusual *Clostridium* species, *Clostridium innocuum* and *Clostridium ramosum*. Many properties of the mycoplasmas were shown to be more similar to the same properties in gram-positive bacteria (e.g., *Bacillus subtilis*) than in gram-negative bacteria (e.g., *Escherichia coli*). These properties include lipid composition (127, 142), 5S rRNA sequences (177), tRNA sequences (117, 176), tRNA gene organization (118, 117), tRNA and rRNA methylation patterns (65), features of aromatic amino acid metabolism (11), specific transport systems (46), and PP_i-dependent enzymes (106). *C. innocuum and C. ramosum* were also shown to be phylogenetically related to the mycoplasmas (119, 180, 185). Neimark and London (103) found that sterol-nonrequiring mycoplasmas evolved from streptococci. Most recently, Fraser et al. (33) got the complete nucleotide sequence of the *Mycoplasma genitalium* genome and compared this genome to that of *H. influenzae*. The *M. genitalium*

genome contains 90 putatively identified genes that do not appear to be present in *H. influenzae*. But almost 60% of these genes have database matches to known or hypothetical proteins from gram-positive bacteria and other *Mycoplasma* species. They also found differences in gene transcription, protein transport, sugar consumption as energy source, and regulatory systems between them. These differences suggest that *M. genitalium* and *H. influenzae* are not phylogenetically related. Hence, the *Lactobacillus* group and the mycoplasmas had a common ancestor and the connection between them should have some biological implications.

The model of mycoplasma phylogeny constructed by Woese, Maniloff, and coworkers is now partially accepted (88) and the knowledge about the mycoplasma phylogeny gives us a better understanding of mycoplasma biology.

Microbial Capsule

All living cells are surrounded by complex carbohydrates (68). On eukaryotic cells, many different carbohydrates are attached as glycoproteins and glycolipids, which play a role in cell-cell or cell-ligand interactions and which are frequently also interacting with extracellular matrix. Polysaccharide capsules of prokaryotes characteristically composed of repeating oligosaccharides, are found on the surface of many bacteria. Both gram-negative and gram-positive bacteria frequently produce capsules. These capsules are commonly composed of only one type of polysaccharide and lie outside the outer membrane of gram-negative cells and the peptidoglycan layer of gram-positive cells (68). Capsules or

capsule-like structures have also been described among several mollicute species. These include *Mycoplasma mycoides subsp. mycoides* (18)], *Mycoplasma dispar* (62), *Mycoplasma gallisepticum* (159), *Mycoplasma hominis* (37), *Mycoplasma hyponeumoniae* (151), *Mycoplasma meleagridis* (51), *Mycoplasma pneumoniae* (183), *Mycoplasma pulmonis* (159) and *Mycoplasma synoviae* (3). Among the *ureaplasmas*, the human pathogen *Ureaplasma urealyticum* expresses capsule (116).

Both capsule and slime are bacterial extracellular polysaccharides. But there are differences between them. As a capsule the polysaccharide is intimately associated with the cell surface and may be covalently bound. In contrast, slime polysaccharides are only loosely associated with the cell surface (149). Distinction between capsule and slime polysaccharides is often operationally defined by the degree of cell association following centrifugation. Differentiation between the two forms may be difficult, since cells producing large amounts of capsule polysacharides may "release" some material at the periphery, giving the appearance of slime production. Mutations from capsule to slime production are known to occur. Most bacteria show a preference for producing one form over another, although some strains of *Klebsiella sp.* can simultaneously produce chemically identical capsule and slime. Several bacteria, including strains of *Rhizobium sp.*, *Agrobacterium sp.*, and *Alcaligenes faecalis* var. *myxogenes* are able to synthesize more than one chemically distinct exopolymer (149).

Capsules are viscous pliable structures, which are hydrophilic and confer a negative charge on the bacterial cells (107, 170). Composed of 99% water, these highly hydrated, polyanionic polysaccharide capsules serve many functions.

These include determining access of molecules and ions to the bacterial cell envelope and the cytoplasmic membrane, the promotion of adherence to the surfaces of inanimate objects or living cells and the formation of biofilms and microcolonies (23). Among certain bacteria, capsules have evolved distinctive structural and functional characteristics which are of cardinal importance in the pathogenesis of infections of animals, humans, plants and insects (147).

Bacterial capsules can be divided into groups based on their chemistry and the conditions under which they are synthesized (148). They may be linear homopolymers of a single chemical substrate, i.e. they are formed entirely of one monosaccharide type. They can be linear heteropolymers, which are composed of two or more monosaccharides. Or they can be multichained or branched polymers composed of two to five monosaccharides and additional moieties (83). Structural diversity arises from a broad range of monosaccharide components and is increased by potential noncarbohydrate substituents and linkage types (71).

The sugar components of capsules can be identified by direct chemical analysis. Some components such as D-glucose, D-mannose, D-galactose and D-glucuronic acid occur very frequently, others such as L-rhamnose or L-fucose are slightly less common, D-mannuronic acid and L-guluronic acid are rare (148).

The production of capsules by the bacterial cell is a very complex process, passing through stages which are associated with different cellular compartments. This process begins with the synthesis of the sugar components of the polysaccharides and their activation by conversion to nucleotide derivatives (31, 182). Nucleotide diphosphate sugars, or more rarely, nucleotide

monophosphate sugars serve as activated donors (167). The enzymes involved in synthesis of the precursors are cytoplasmic, but may in some cases be loosely associated with the cytoplasmic membrane (150). The subsequent polymerization is catalyzed by an inner membrane bound transferase complex. These transferases are poorly defined, with an unknown number of components and a catalytic mechanism which remains obscure (67, 149, 165, 167). But some neutral polysaccharides produced by gram-positive cocci, such as the levans and glucans, are synthesized from sucrose on the outer surface of the prokaryotic membrane (148).

Polymerization is generally believed to involve a lipid carrier. The lipid involved may be undecaprenol phosphate on which monosaccharides or oligosaccharides are assembled. The precise role played by the lipid is presently unknown (10). Some possibilities include facilitating the structure and ordered formation of the repeating unit structure, solubilization of hydrophilic oligosaccharides in a hydrophobic membrane domain, transport across the membrane, regulation of priorities in polysaccharide synthesis on the basis of lipid availability, or a combination of these events (148, 167). The final polymer is transferred from the lipid carrier to an endogenous acceptor (141, 167). It is suggested that the acceptor has phospholipid and this phospholipid may function as an anchor for extracellular polysaccharides in the outer membrane (47, 126). The export of capsular polysaccharide consists of at least two stages, viz., the translocation across the cytoplasmic membrane and subsequent transport to the cell surface (31). The final step is the organization of polysaccharide into a capsule. The translocation and the organization is very poorly defined. The regulation of

capsule synthesis, involving either plasmid, or chromosomal genes, or both, has been the subject of a series of studies (90). In *Rhizobium meliloti*, capsule genes are divided between the chromosome and the megaplasmid pRmeSU47b (84), but in most other bacteria the determinants are chromosomal. In *E. coli* K1, the capsule genes are clustered at the *kps* locus. The genes determining the expression of *E. coli* group I and group II capsular polysaccharides are located at different sites on the bacterial chromosome (105, 171). It was shown that these gene clusters do not hybridize *in vitro*. Recent advances in the genetic analysis of group II and related capsules, especially with *E. coli*, *N. meningitidis*, and *H. influenzae* (14, 15, 77, 78, 131, 143, 173) have revealed the organization and function of these capsule genes. It was found that the capsule genes of *E. coli* are organized in three regions. The central region 2 directs the polymerization of the respective capsular polysaccharide; it is thus specific for a given capsule; region 2 is flanked by region 3, which directs the translocation of the polysaccharide across the cytoplasmic membrane, and region 1, which directs the transport through the periplasm and the outer membrane into capsular compartment. Both region 1 and 3, which serve general functions, can be exchanged between different *E. coli* with group II capsules. It was found later that region 1 also encodes other functions than those of transport. In other bacteria such as *Xanthomonas campestris* (57, 163), the capsule genes may be organized into more than one cluster. Recombinant DNA technology has helped resolve the basis for genetic instability of the capsule trait in some bacteria. In *H. influenzae* type b, capsule genes are present as a tandem repeat. Homologous recombination events resulting in the loss of one copy of the genes may lead to

the appearance of unencapsulated bacteria (59).

Control of precursor availability by either feedback inhibition (76) or degradation (172) have been reported. Regulation of capsular synthesis at the level of lipid intermediate availability has also been proposed (148). For the most part, however, regulatory elements acting on capsular synthesis have not been defined.

Capsules have long been associated with virulence properties of bacteria. More recently it has been possible to define at the molecular level many of the mechanisms involved in the virulence provided by bacterial capsules.

Most capsular polysaccharides are hydrophilic and confer a negative charge on the bacterial cell, characteristics which are intrinsically antiphagocytic in their effect. It is argued (107, 170) that the hydrophilic properties of polysaccharide capsules act by reducing the surface tension at the interface between the phagocytic cell and the bacterium and that this impairs the ease with which phagocytic ingestion occurs. Considering surface charge, it seems reasonable that contact between capsulated bacteria and phagocytic cells would be compromised since the net negative charge on each would tend to result in mutual repulsion.

The interaction of bacterial capsules with the complement system is an important mechanism with which tissue-invasive bacteria can evade the nonspecific host defenses. As far as we know today, it is the interference of capsular polysaccharides with regulatory proteins that upsets complement action. A pivotal compound, joining both pathways, is factor C3. Its conversion to the active form C3b is activated by the classical convertase C2bC4b, which is under

the control of a C4 binding protein, C4bp (104). Some capsular polysaccharides appear to bind C4bp and thus interrupt the complement cascade (24). Activation of the central component C_3 to C_{3b} by the alternative pathway is controlled, *inter alia*, by factors B and H. These regulate the affinity of C_{3b} to bacterial cell systems. Some capsules decrease the affinity of factor B to C_{3b} and others increase its affinity to factor H (24, 87). In both cases, the surface deposition of complement is decreased and the encapsulated bacteria escape the bactericidal activity of the complement system. A somewhat simpler interpretation, which may be given in addition to the above, is that encapsulated bacteria bind the complement components too far away from the cell surface to be harmful (122, 24).

Although bacterial capsules generally induce the formation of anticapsular antibodies, which activate host defenses, their immunogenicity is often age related (115). Thus, many encapsulated bacteria are not immunogenic in newborns or young children (69). In general, infants aged less than about 2 years possess low or are absent concentrations of anticapsular antibodies even following recovery from systemic infection with capsulated bacteria. The reason for the delay in the ability to respond to capsular polysaccharides is due to the delayed maturation of Lyb-5 B cells. It is generally argued that young infants in whom clearance of tissue-invasive bacteria is least efficient face a period of heightened susceptibility starting from the point at which levels of maternally acquired antibodies decline at about 3 months until the latter half of the second year, when the ability to mount endogenous, type-specific antibodies against polysaccharide antigens matures.

Certain bacterial capsules are not or only poorly immunogenic also in adults. Structural elucidation of these capsules has revealed that this is due to identity of the capsules with certain substances found in the host. For example, The K1 capsular polysaccharide of *E. coli*, a poly-α-2,8-sialic acid, has the same structure as the carbohydrate terminus of the embryonic neural cell adhesion molecule n-CAM (32). By the same token, the *E. coli* K5 capsular polysaccharide, a heteropolysaccharide consisting of 4-β-glucuronic acid and 4-α-N-acetylgluosamine (169), is identical with the first polymeric intermediate of heparin (100). These findings emphasize that capsules counteract host defenses not only by interaction with complement components but also by evasion of immune recognition owing to molecular mimicry.

Finally, the shedding of capsule from the bacterial surface is a potential mechanism for jettisoning attached host factors or for nullifying the functional role of circulating host factors such as type-specific antibodies (97).

Polysaccharide Binding Proteins

Proteins and enzymes that bind carbohydrates are present in large numbers in all living cells and are involved in a myriad of important biological functions (109). This abundance is primarily due to the fact that carbohydrates derived from carbon dioxide fixation constitute the bulk of the organic matter on the earth, and together with their various derivatives and polymeric forms are utilized for many essential purposes in the cell. For example, prokaryotic capsules that are polysaccharides play a very important role in the survival of bacteria in their

hosts. Some proteins bound to capsular polysaccharide are shown to be associated with capsule production. Currently, most of the references about CBP are focused on the glycosyltransferase enzymes of gram-positive cocci that have polysaccharide-synthesis and binding activities. Therefore this review is mainly focused on the general properties of these enzymes. In addition, there will also be a short review about glucan-binding proteins.

Glucosyltransferases

Streptococcus mutans and its related oral streptococci are microorganisms that synthesize adherent water-soluble and/or water-insoluble glucans from sucrose by glucosyltransferases (GTFs). The complex polysaccharides have been considered important for colonization of the bacteria on tooth surfaces (53, 98), which promotes enamel demineralization and attendant dental caries (86). It was first shown by Guggenheim & Newbrun (52) that a single strain could produce a number of electrophoretically distinct GTFs. Ciardi et al. (22) showed that some of these enzymes synthesized soluble polymers and others synthesized insoluble polymers. Since then several laboratories have isolated and characterized multiple GTFs from various *Streptococcus mutans* strains (38, 45, 129, 189). These GTFs are generally classified into three groups: GTF-I, which produces a water-insoluble, primarily α-1,3-linked glucan; GTF-S, which produces a water-soluble, primarily α-1,6-linked glucan; and GTF-SI which produce a combination of water-soluble and -insoluble glucans (44). In *S. mutans*, GTFs are encoded by three genes, *gtfB*, *gtfC*, and *gtfD*, which express different enzyme activities: *gtfB* encodes GTF-I, *gtfC* encodes GTF-SI, and *gtfD* encodes GTF-S (7, 36, 55, 56). The insoluble glucan produced primarily by GTF-I

and GTF-SI mediates the sucrose-dependent attachment of *S. mutans* to the smooth surfaces (53). The contribution of the water-soluble glucan to the dental caries process is unknown, but it causes aggregation of certain bacteria and can serve as an extracellular energy store (44). Generally, GTF-S is found in the culture medium and GTF-I is cell associated (57). GTF-I and GTF-SI have a high degree of amino acid sequence identity (19). But GTF-I and GTF-S, which synthesize distinct glucan products, are also structurally different from each other (92, 129, 133). However, since GTFs catalyze similar processes involved in glucan synthesis, it is likely they share many structural features relevant to the catalysis. Although the pathway of glucan synthesis is incompletely determined, several investigators have suggested that GTF protein is composed of domain structures bearing distinct functions, such as glucosyl transfer and glucan binding(28, 74, 93).

The binding potential of GTF to glucans is suggested by several studies (40, 53, 91). In the meantime, the structure and function of GTFs have been studied by biochemical and recombinant DNA techniques. Ferretti et al. (28) were the first to determine the complete nucleotide sequence of the GTF-I gene from a strain of *Streptococcus sobrinus* (MFe28) and have located a glucan-binding site by deletion analysis of Gtf-I in the carboxy-terminal region of the GTF-I protein, which is essential for the enzyme activity. Furthermore, Mooser and Wong (93) isolated a glucan-binding fragment from a trypsin digest of GTF-S and Kobayashi et al. (75) also isolated a 55 kDa peptide from a trypsin digest of GTF-I which has a glucan-binding domain. These observations indicate that both GTF-I and GTF-S proteins contain a structural domain which functions in glucan binding. The

location of the glucan binding region in GTF-I has been narrowed to the C-terminal one third of the enzyme (28). These glucan-binding domains are composed of a series of repeated sequences that have been classified into four different classes (A-D) by virtue of sequence similarity and which, by inference, have been suggested to be of functional importance (1, 8, 28, 41, 42, 43, 44, 60, 130, 168). Recently, a relationship between these repeats and those of the ligand-binding repeats of toxins A and B of *Clostridium difficile*, a glucan-binding protein (GBP) from *S. mutans* and the lysins of *Streptococcus pneumoniae* have been observed both immunologically and at the amino acids sequence level (44, 139, 186, 187) , suggesting that sequences of this type may play a role in the biology and/or pathogenicity of a range of Gram-positive bacteria. The glucan-binding proteins (GBP) from *S. mutans* contain two sets of non-adjacent repeats (5.5 A and 4 C repeats) which span almost the entire length of the protein (8) (Note: two series of repeats were identified within the GBP and were designated A and C repeats; the A repeats were represented by five regions of 32 to 34 amino acids, with a partial sixth repeat of 12 amino acids at the C terminus of the protein, hence 5.5 A; the C repeats ranged from 17 to 20 amino acids and occurred four times throughout the protein sequence.). The toxin A class II B-repeat consensus sequence shares 14 of 16 consecutive residues with the consensus class A repeats from GBP (186). Amino acid repeats similar to the class A and C repeats of *S. mutans* GBP also occur in GTF enzymes. Genes for four enzymes have been sequenced: two are from *S. mutans* GTF-B (6 A and 5 C repeats) (130) and GTF-C (3.5 A and 2 C repeats) (168) and two are from *Streptococcus downei*, GTF-I (6 A and 5 C repeats) (28) and GTF-S (4.5 A and 4

C repeats) (44). Russell (123) has suggested that an appropriate single-base frameshift in the termination codon of the gene sequence reported for GTF-C results in 5 A and 3 C repeats. The A and C repeats show little similarity to each other and the relative positions of the C repeats vary between the GBP and GTF. The comparison of the amino acid sequences of all group A repeats from the GBP, GTF, and those of the two clostridial toxins reveals that three amino acids are conserved in almost all cases, the tyrosine-phenylalanine dipeptide, and a glycine 10 residues upstream of the tyrosine-phenylalanine dipeptide (186). Aromatic residues (tyrosine, phenylalanine and tryptophan) are generally conserved at certain other positions, particularly in the two positions just upstream of the tyrosine-phenylalanine dipeptide. Charged and polar residues (particularly lysine, glycine and threonine) intermingled with turn-promoting residues (proline, glycine, asparagine and serine) predominate at other sites within the repeats. These repeats favor the protein-polysaccharide complexes of GTF.

The analysis of glucosyltransferase glucan-binding regions and related sequences by Giffard et al. (43) extends these previous studies by defining a fundamentally conserved repeating motif that transcends the boundaries of all previously designated repeat structures that have been found in the related carboxyl-terminal ligand-binding domains of streptococcal GTFs, *C. difficile* toxin A, and *S. pneumoniae* autolysin, as well as glucan-binding protein. Results have shown that while in any given GTF-pair these motifs may posses a wide range of similarity, certain residues are highly conserved. A common rationale used by workers for classification of repeats into different classes is that this may aid in

accounting for differences in the binding properties of the ligand-binding domains from different enzymes.

The glucan-binding domain has been cloned, proteolytically excised from the native enzyme and partly characterized. In contrast, there is much less work reported about the catalytic domains of the GTFs. The location of the catalytic sites was done by deleting amino acids from the N-terminal region. The study of GTF-I from *S. sobrinus* showed that the N-terminal 80 amino acids were nonessential for enzymatic activity. However, the loss of 260 amino acids from the N-terminus of GTF-I by further deletion diminished the enzymatic activities for sucrose splitting and glucan synthesis, although glucan binding was not affected because of an intact C-terminus (1). Furthermore, it has been shown that antiserum to a chimeric protein composed of 15 amino acids of the N-terminus of GTFB (residues 342 to 356) inhibited the water-insoluble glucan synthesis of a crude enzyme prepared from a culture supernatant of *S. mutants* GS-5 (25). Then, an active site responsible for sucrose binding was proposed after sequencing of a peptide from the stabilized glucosyl-enzyme complex (94, 95) and confirmed by site-directed mutagenesis (70). This peptide of nine amino acids is located in the N-terminal one-third of the GTFs. Together, these results suggested that the N terminus of the GTFs may play a central role in the enzymatic activities for sucrose splitting and glucan synthesis.

An amino-terminal region of approximately 40 residues extending from positions 387 to 427 of GtfB (or 413 to 453 of GtfC) was identified to be highly conserved among the GTFs of several streptococci (21). Furthermore, the DNA sequence variations in this region did not alter the amino acid sequence (20),

suggesting some biological importance of this conservation. A 19-amino-acid peptide containing residues 435 to 453 of GtfC was able to reverse the inhibitory effect of a polyclonal antibody which neutralized GTF enzymatic activities (20). Monoclonal antibodies against the 19-amino-acid peptide recognized the conserved region and inhibited insoluble glucan synthesis by GtfC and the attachment of *S. mutans* to glass surfaces (19). These results indicate that the N-terminal conserved region of a GTF is functionally important for both enzymatic activity and bacterial adherence.

It has recently been suggested that intra- and interspecies variation between streptococcal enzymes may be an ongoing process that could circumvent the idea of using conserved epitopes as immunogens (43). Because of the central role that GTFs play in dental plaque formation, they have been considered as potential components of a dental caries vaccine (154). Experiments with animal models and with humans have supported this potential. For example, GTFs from *S. sobrinus* and *S. mutans* of the mutans streptococci have been demonstrated to elicit immune responses which are protective against experimental caries caused by infection with several mutans streptococcal species (136, 137, 155). The salivary immunoglobulin A (IgA) antibody response to oral and/or local administration of GTFs to humans has been significantly correlated with interference with reaccumulation of indigenous mutans streptococci (134, 138). Although the basis for experimental protection with GTF-based vaccines is unknown, it is likely to involve antibody mediated inhibition of the catalytic and/or the glucan-binding activity of GTF (157, 158). Although antibody binding to functionally important domains may not be necessary theoretically to achieve

immunological protection, binding to catalytic domains should result in loss of enzyme activity. Antibody-mediated inhibition of these functional activities has been demonstrated (157, 158).The peptide DANFDSIRVDAVDNVDADLLQ (CAT) contains an aspartic acid that has been implicated in an early catalytic step of glucan synthesis (94, 95). Rat serum antibody and mouse IgM MAb to this peptide significantly inhibited the ability of *S. sobrinus* GTF to synthesize glucan (140). The immunogenicity and antigenicity of a multiply antigenic peptide construct containing four copies of the synthetic peptide TGAQTIKGQKLYFKANGQQVKG (GLU) were measured in rodents and humans (139). This peptide sequence was derived from a pattern of repeating sequences which have been identified in the C-terminal in all GTFs from mutans streptococci and have been associated with glucan binding properties of GTF (1, 29, 53, 56, 86). Antibody to this construct reacted with native GTF of both *S. sobrinus* and *S. mutans* and inhibited its function. This antibody could also modify colonization of the oral cavity by mutans streptococci in ways other than by enzyme inhibition. Taubman et al. (156) demonstrated that immunization of rats with peptide constructs GLU and CAT resulted in significantly reduced smooth surface and sulcal caries after infection with *S. sobrinus*. Thus, antibody to epitopes present in the N-terminal and C-terminal domain regions of GTF apparently can interfere with enzyme function. In addition, Dertzbaugh and Macrina (25) have demonstrated inhibition of GTF activity with antibody to a chimeric peptide that included a sequence closer to the N terminus (residues 342 to 356) than the CAT sequence (residues 487 to 507) in *S. mutans* (60, 168). Also, Chia and coworkers (20) have synthesized a 19-mer peptide, homologous to a sequence nearly

adjacent to the CAT region, that contained an epitope able to remove some of the GTF-inhibitory activity of polyclonal antibodies directed to *S. mutans* GTF-B and GTF-C. These observations suggest that inhibition of GTF activity can be achieved by the binding of antibody to sequences that may not necessarily be associated with function.

The above mentioned studies suggest that subunit vaccines that are based on GTF sequences have the potential to modify dental caries caused by mutans streptococci. Antibody-mediated inhibition of the caries process could be achieved by interference with GTF catalytic activity, by modification of the nature of the glucan product, or by agglutination of microorganisms through bacterial cell surface GTF epitopes. The success of such a vaccine strategy is predicated on the incorporation of an appropriate peptide into a carrier construct that results in an effective immune response.

In summary, GTFs have been implicated as one of the virulence factors in the pathogenicity of mutans streptococci. GTFs can synthesize glucans from sucrose and concomitantly bind these glucans. This ability of GTFs is thought to contribute to cell adherence and accumulation on tooth surfaces. GTFs have glucan-binding domains, which are located in the C-terminal one-third of the GTFs, and catalytic domains, which are located in the N-terminal one-third of the GTFs. The antibodies against fragments bearing these domains are demonstrated to protect the tooth from forming dental plaque. GTFs are potential vaccine candidates.

Glucan-Binding Proteins (GBP)

In addition to the GTFs, there is another group of proteins synthesized by

mutans streptococci that can also bind glucans. This group of proteins are called glucan-binding proteins, and they do not have GTF enzymatic activity.

Several GBPs of different molecular weights have been reported. Russell (124) isolated a 74-kDa GBP from the extracellular fluid of cultures of *S. mutans* serotype C. Landale and McCabe (82) purified a 15-kDa extracellular GBP from *S. sobrinus*. Wu-Yuan and Gill (188) described a 87-kDa CBP synthesized by *S. sobrinus* B13. Smith et al. (135) reported a 59-kDa GBP of *S. mutans*. Western blot analysis showed that the 87-kDa GBP was antigenically related, but not completely identical, to the 74-kDa GBP (188). Smith et al. (135) also demonstrated that antiserum to 74-kDa GBP could recognize epitopes on *S. sobrinus* 87-kDa GBP. The 59-kDa GBP did not cross-react with 74-kDa CBP. Banas and coworkers (8) have determined the sequence of DNA coding for a GBP from *S. mutans* Ingbritt. Interestingly, the molecular mass of the processed protein was calculated to be approximately 59 kDa, although the product of the *gbp* clone was shown to react with antisera to the 74-kDa GBP in Western blots. These results suggests some structural relationships between GBPs of different streptococcal species.

Although all of these mutans streptococcal GBPs display affinity for glucans rich in α-1,6-glucosyl linkages, they do not have GTF enzymatic activity. But the 74-kDa GBP from *S. mutans* was shown to have fructosyltransferase (FTF) activity (125). Despite the fact that both FTFs and GBPs synthesize fructans from sucrose in *S. mutans* and show closely similar electrophoretic mobilities and isoelectric points, the restriction maps of the two genes are distinct, their DNAs do not hybridize and there is no immunological cross-reaction between the two

proteins (2). There is no report of the fructosyltransferase activity of the other GBPs. The relationship between GBP and FTF therefore remains unclear.

Smith et al. (135) demonstrated that antisera to the 74-kDa GBP and to the 59-kDa GBP of *S. mutans* reacted weakly with *S. mutans* and *S. sobrinus* GTFs in Western blot assays. Wu- Yuan and Gill (188) and Russell (9) showed that antisera to *S. mutans* 74-kDa GBP cross-reacted with GTFs from *S. sobrinus* and *S. downei*. Banas and coworkers (8) have determined the sequence of DNA coding for a GBP from *S. mutans* Ingbritt. The most striking feature of the deduced amino acid sequence was the presence of two sets of repeats that spanned three-quarters of the length of the protein. The A repeats were similar to the A repeats identified in GTF-I of *S. downei* (28), and the GBP C repeats shared partial identity to regions between the A repeats. Both the A and C repeats of GBP overlapped reiterated sequences identified by Kuramitsu and colleagues within the proteins encoded by *gtfB* (130) and *gtfC* (168), although the regions homologous to the GBP C repeats were consistently located between regions similar to the GBP A repeats. The significance of the 11-amino-acid sequence from GTF-I that is repeated 15 times in GBP with varying degrees of identity is uncertain; however, the beginning of the 11-amino-acid repeats coincides with the beginning of the A and C repeat regions. Portions of the GBP A repeats were also similar to a repeat region within two pneumococcal autolysins. The repeats in autolysins were within the carboxy-terminal portion of each protein, and there is evidence that the carboxy-terminal half of each protein functions in substrate recognition (39). It would appear that the repeat domains have been duplicated and preserved among several genes in different species

during evolution, indicating some importance in structure, function, or both.

When a consensus amino acid sequence of the A repeats of the GBP was compared with similar sequences in the GTFs, certain amino acids were found to be conserved throughout. These were most often glycine, but a tyrosine-phenylalanine pair was also conserved. Other amino acids that were conserved in almost all instances included threonine, tyrosine, asparagine, glutamine, and lysine. All of these amino acids, with the exception of phenylalanine, have been reported to be involved in hydrogen bonding in protein-saccharide complexes (109).

There is some evidence that the GBPs may be involved in the formation of cohesive plaque. Russell et al. (125) demonstrated that a GBP-deficient mutant of S. *mutans* forms loosely adherent plaque in vitro. GBPs from *S. sobrinus* (82) and *S. cricetus* (26) are also thought to contribute to adherence and accumulation of the organisms in plaque. The sequencing of the *gbp* gene has laid the foundation on which further experimentation can build an understanding of the significance of the repeating regions in GBPs and ultimately of the role of the GBP in *S. mutans* virulence.

Other organisms that produce capsules may also have GTF- and GBP-like proteins. By investigating these proteins and comparing them with GTFs and GBPs we may increase understanding of capsule production and provide some hints on how to control the diseases caused by these organisms. Therefore, it is very important to investigate this kind of proteins in organisms other than streptococci.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma (St Louis, Missouri) unless otherwise indicated.

Mycoplasma

The SDO strain of *Mycoplasma dispar*, initially isolated from a pneumonic calf (164), was cloned twice and used at passage level of 10 or 11. The mycoplasma was grown in modified Friis broth (73) with 20% horse serum (HyClone, Logan, Utah).

Preparation of Capsule-coated Agarose-bound RCA_{120}

RCA_{120} (agarose-bound Ricinus Communis Agglutinin I, Vector, Burlingame, California) bead slurry was washed three times in ice-cold capsule buffer (10 mM Hepes pH 8.5, 0.15 M NaCl, 0.1 mM $CaCl_2$) by centrifuging at 14,000 x g for 3 minutes at 4°C. Purified capsule (from Dr. Rosenbusch's Lab, Iowa State University) was added to the beads and reacted for 1 hour at room temperature with tumbling. Then the beads were washed five times in ice-cold capsule buffer by centrifugation.

Purification of Capsule-Binding Protein

M. dispar cells were grown in Friis broth with 20% horse serum at 37°C with shaking for 19 hours. The mycoplasma cells were harvested from the culture and washed three times by centrifugation in PBS at 27,000 x g, for 20 minutes at 4°C. Capsule polysaccharide was extracted from the washed cells by incubating in PBS at 56°C for 20 minutes. The mycoplasma cells were then recovered by centrifugation at 27,000 x g, for 20 minutes at 4°C. The cells were resuspended in ice-cold lysis buffer (capsule buffer with 1.5% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 1 µM leupeptin). The suspension was homogenized in a tissue grinder, and DNAase I was added to digest DNA at 4°C for 1 hour with tumbling. Non-extractable materials were removed by centrifugation at 14,000 x g, for 5 minutes at 4°C. The supernatant was reacted with capsule-coated agarose-bound RCA_{120} for 1 hour at 4°C plus 5 minutes at room temperature with tumbling. Then the beads were washed three times in ice-cold capsule buffer with 0.5% Tween 20 by centrifugation at 14,000 x g, for 3 minutes 4°C. The washed beads were mixed with the same volume of 2X denaturing buffer (final concentration, 0.15 M Tris pH 6.8, 0.8% SDS, 2% glycerol, and 0.5% ß-2-mercaptoethanol) and boiled 2 minutes before loading the gel. Discontinuous PAGE was performed with SDS as described previously by Laemmli (81). The gels were made with 7.5% separating gel and 4% stacking gel. A Hoeffer SE 600 series vertical slab unit was used to electrophorese protein samples (Hoeffer, San Francisco, California). The protein samples were prepared in 40 µl solution, mixed with 40 µl 2X denaturing buffer, and boiled for 2 minutes.

Then the protein samples were loaded into the wells of 1.5 mm-thick gels. The gels were run at 80 mA (constant current) at room temperature for about 1.5 hour. A set of known molecular weight standards (molecular weight range 14,300-220,000, Amersham, Arlington Heights, Illinois) was used to estimate the molecular weight of the separated proteins on the SDS-PAGE gels.

Radiolabeling of Mycoplasma Cells and Purification of Labeled Capsule-Binding Protein

To metabolically label mycoplasmas, 20 ml (total protein=1120 μg) of 19 hour broth culture was centrifuged at 27,000 x g, for 20 minutes at 20°C. The pellet was washed once with labeling medium (Hanks balanced salts solution with 85 mM Hepes pH7.4, 1% bovine serum albumin, and 50 μg/ml bacitracin) . The pellet was suspended in 4 ml labeling medium with 100 μCi of L-[^{35}S] methionine (5 mCi/ml, Amersham, Arlington Heights, Illinois) per ml and incubated at 37°C for two hours. The labeling was stopped by adding 20 ml 37°C labeling medium with 100 mM unlabeled methionine and incubated at 37°C for 30 minutes. The cells were harvested by centrifugation, and washed twice with PBS containing 1 mM PMSF. The CBP was purified as described above. After electrophoresis, the gel was stained in 100 Coomasie Blue with rocking at room temperature overnight. The gel was destained with changes of Destain I (50% methanol, 10% acetic acid) for 2 hours, and then with Destain II (30% methanol, 3% acetic acid, 3% glycerine) for 30 minutes. After destaining, the gel was treated with 100 ml 1 M sodium salycilate for 30 minutes, and then dried in gel dryer. Autoradiography

was performed at -70°C using XAR-5 film in casettes with enhancer screens (Kodak, Rochester, New York).

Preparation of Rabbit Antiserum against Capsule-Binding Protein

M. dispar was grown in 300 ml Friis broth with 20% horse serum at 37°C for 19 hours. CBP was separated from the cell lysate by following the above procedure. Gel slices of 2 mm containing bands separating at 126 kDa were obtained from a preparative gel and crushed with 2 ml PBS by extrusion through 18G and then 20G needles. The crushed gel was injected subcutanously into each of two rabbits using 1 ml per rabbit. Booster injections were given three times every ten days with the same amount of freshly prepared gel slices. Ten days after the fourth inoculation, the rabbits were bled and the serum was collected and stored at -20°C.

Preparation of Globulin Fraction of Rabbit Antiserum

Equal volume of ice-cold saturated $(NH_4)_2SO_4$ (pH 7.8) was added dropwise to ice-cold rabbit serum by stirring constantly. The suspension was stirred overnight at 4°C, then centrifuged at 12,100 x g for ten minutes at 4°C. The pellet was resuspended to original serum volume in ice-cold saturated $(NH_4)_2SO_4$ (pH 7.8) diluted 1:1 with 0.85% NaCl. The suspension was stirred for 1 hour at 4°C and centrifuged as above. The pellet was resuspended, stirred, and centrifuged again as described above. The pellet was resuspended to 1/5 original serum

volume in pH 8.6 TT solution (24 mM Tricine, 81 mM Tris, and 1 mM calcium lactate with 0.85% NaCl) and dialyzed against the same solution at 4°C for 72 hours with 3 changes of dialyzant. The globulin fraction was harvested and stored at 4°C with 0.02% sodium azide.

Triton X-114 Phase Partitioning of Proteins

Mycoplasmas from 19-hour cultures were subjected to TX-114 phase partitioning by a slight modification (121) of the method originally described by Bordier (13). Briefly, mycoplasma cells were suspended in Tris-HCl buffer (pH 7.4) containing 1% (vol./vol.) precondensed TX-114, 150 mM NaCl, and 1 mM PMSF and were incubated at 4°C for 30 minutes. This preparation was centrifuged at 4°C for 5 minutes at 10,000 x g to remove insoluble materials. The supernatant was transferred to a new tube , incubated at 37°C for 5 minutes, and then centrifuged at 22°C for 5 minutes at 10,000 x g. The resulting heavy, detergent-enriched fraction (TX-114 phase) and a lighter, detergent-depleted fraction (aqueous phase) were placed in separate tubes, readjusted to a concentration of 1% (vol./vol.) TX-114, and repartitioned as described above. After three cycles of phase fractionation for the detergent phase and for aqueous phase, the condensed detergent-enriched phase was used in parallel with the aqueous phase for subsequent electrophoresis and immunoblot analysis.

Trypsin Treatment of Intact Mycoplasma Cells

Intact organisms from fresh broth cultures were treated with trypsin by variation of a previously described method (72, 114, 121). Briefly, mycoplasmas from 19-hour culture were harvested by centrifugation, rinsed twice with PBS (pH 7.2), then rinsed with PBS (pH 8.0). Cells were incubated at 37°C for 1 hour in PBS (pH 8.0) containing varying concentrations of L-1-tosylamide-2-phenylethyl-chloromethyl ketone-trypsin (TPCK trypsin) per ml. Mycoplasma cells were sedimented by centrifugation at 4°C. Pelleted cells were washed twice with PBS containing 1 mM PMSF. Then the cells of each concentration of trypsin treatment were divided into two equal parts. One part was directly applied to subsequent electrophoresis and immunoblot analysis. The other part was lysed with lysis buffer as described previously and then reacted with capsule-coated RCA_{120} beads. The washed beads were then applied to electrophoresis and immunoblot analysis.

Purification of Binding-domain-bearing Tryptic Digestion Products from the Supernatant

Intact cells were treated by TPCK-trypsin as described above, but only at the concentration of 500 µg per ml. Mycoplasmas were spun down at 4°C, and the supernatant was collected. After addition of 1 mM PMSF the supernatant was divided equally into two parts. One part was concentrated by ultrafiltration on 30,000 dalton membranes (PM30) (Amicon, Lexington, Massachusetts) to 50 µl,

and applied to electrophoresis and immunoblot analysis. The other part was reacted with capsule-coated RCA_{120} beads as described above. Then the washed beads were applied to electrophoresis and immunoblot analysis.

Carboxypeptidase Y Treatment of Intact Mycoplasma Cells

Intact mycoplasma cells were treated with carboxypeptidase Y following a method previously described (58, 121). Briefly, mycoplasmas from a 19-hour culture were harvested by centrifugation, rinsed twice with PBS (pH 7.2), then rinsed with PBS (pH 6.0). The washed cells were divided equally into two parts. One part was incubated at 37°C for 1 hour in PBS (pH 6.0) containing 1 mg/ml carboxypeptidase Y (Boehringer Mannheim, Indianapolis, Indiana). The other part was set as control. Mycoplasma cells were sedimented by centrifugation at 4°C. Pelleted cells were washed twice with PBS containing 1 mM PMSF. Then the washed cells were applied to electrophoresis and immunoblot analysis.

Immunoblotting

The western blot technique described previously (166) was used to detect transferred proteins with some modification. The proteins eletrophoresed on the SDS-PAGE gels were transferred onto Zeta-Probe blotting membranes (Bio-Rad, Hercules, California) by using an electrotransfer apparatus (Bio-Rad, Hercules, California). Briefly, the gels were rinsed three times for 10 minutes each in the transfer buffer (25 mM sodium phosphate buffer, pH 6.8) without methanol before

setting up the sandwich. Transfer was performed at 0.19 A overnight with water cooling. After transfer the membrane was incubated in washing buffer (PBS with 0.1% Tween 20) at 60°C for 1 hour. Then the membrane was blocked by immersion in 5% skim milk in washing buffer at room temperature for 1 hour or at 4°C overnight with shaking. The membrane was extensively washed three times for 15 minutes each with washing buffer at room temperature with shaking. The membrane was incubated with 1:20 polyclonal rabbit-anti-CBP globulin at room temperature for 2 hours or at 4°C overnight with shaking and then washed three times as above. Peroxidase-conjugated goat-anti-rabbit IgG at 1:1500 (diluted in washing buffer) was added and incubated at room temperature for 2 hours or at 4°C overnight with shaking. The membrane was again extensively washed as above. Enzyme substrate prepared immediately prior to use was poured onto the membrane, and incubated at room temperature for about 5 minutes with shaking. Reaction was stopped by rinsing the membrane in water. Photography was done by using Kodak Tmax 100 professional film (Kodak Eastman, Rochester, New York).

RESULTS

SDS-PAGE Analysis of L-^{35}S Methionine Labeled Capsule-Binding Protein

Mycoplasma cells were labeled with L-^{35}S methionine. Proteins captured on capsule-coated RCA_{120} beads were analyzed with SDS-PAGE (Fig. 1). Many proteins that were labeled could be captured by the capsule-coated RCA_{120}. The 126 kDa band was prominent and was the largest one.

Immunogenicity of Capsule-Binding Protein in Rabbits

The pooled sera of rabbits injected with purified CBP from *M. dispar* were tested by Western blot analysis for antibody activity (Fig. 2). The antibodies reacted with a 126 kDa band. There was also weak reactivity with a 200 kDa band , but it disappeared when the dilution of serum was increased. Reactivity with the 126 kDa band was detected up to a 1:320 dilution of the globulin fraction. There was no reaction between the sera from the rabbits before immunization with CBP (data not shown). The antibodies also reacted with purified CBP recognizing only a 126 kDa band (Fig. 3).

Identification of Capsule-Binding Protein as Integral Membrane Protein

To evaluate the partitioning characteristics of the CBP, TX-114 phase, aqueous phase of fractionated organisms, and whole organisms without

fractionation were separated by SDS-PAGE and immunoblot was done with antibodies against CBP (Fig. 4). In the TX-114 phase, the 126 kDa protein was recognized by the antibodies against CBP and the band was very strong. In the aqueous phase, a very faint 126 kDa band was also visualized. This provided indication that the CBP was primarily partitioned into the TX-114 phase.

Surface Location of Capsule-Binding Protein

To determine if the Capsule-Binding Protein was located on the external surface of *M. dispar* cells, freshly harvested, intact cells were treated with graded amounts of TPCK-trypsin. Sets of TPCK-trypsin-treated cells were subjected to SDS-PAGE and immunoblotted with antibodies against CBP (Fig. 5). The addition of various amounts of TPCK-trypsin resulted in the generation of epitope-bearing degradation products with molecular masses of 110 kDa, 100 kDa, 95 kDa, and 56 kDa.

Tryptic Digestion Product Bearing Capsule-binding Domain

CBP was defined by the previous experiment as an integral membrane protein. To locate the capsule-binding domain, mycoplasma cells were treated with trypsin and then lysed prior to capture of capsule-binding domains on capsule-coated RCA_{120} beads. Capsule-binding domains could only be shown as full-length CBP if associated to the mycoplasma cells (Fig. 6). This is evidence that trypsin treatment removed the capsule-binding domains from membrane-

anchored CBP.

Purification of Capsule-binding Fragments of Capsule-Binding Protein

The fragments of CBP associated with the mycoplasma cells after trypsin digest of the intact mycoplasma cells did not bear the capsule-binding domains. Therefore, fragments from the supernatant were examined for capsule-binding activity. The supernatant from the trypsin digest of intact mycoplasma cells contained bands of 66 kDa, 63 kDa, 61 kDa, 44 kDa, and 39 kDa. Of these, only the 66 kDa band was shown to have capsule-binding activity (Fig. 7). The 66 kDa bands from total supernatant or capsule-binding fraction of supernatant had the same density, indicating that the 66 kDa fragment was totally captured by capsule and had no internal trypsin-cleavage sites. There was an unexpectedly strong reactivity of the anti-CBP antibody with a 39 kDa fragment that does not bear a capsule-binding domain.

Orientation of Capsule-Binding Protein Anchored in the Membrane

The orientation of CBP was characterized by digestion of intact organisms with carboxypeptidase Y (Fig. 8). The digestion yielded a ladder of epitope-bearing products with the same spacing, and a minimum size of 60 kDa.

Figure 1. SDS-PAGE analysis of L-^{35}S methionine labeled M. dispar proteins captured on capsule-coated RCA_{120} beads. Standard molecular weights are shown on the left side. The 126 kDa capsule-binding protein is indicated on the right side. (Exposure time: 72 hours)

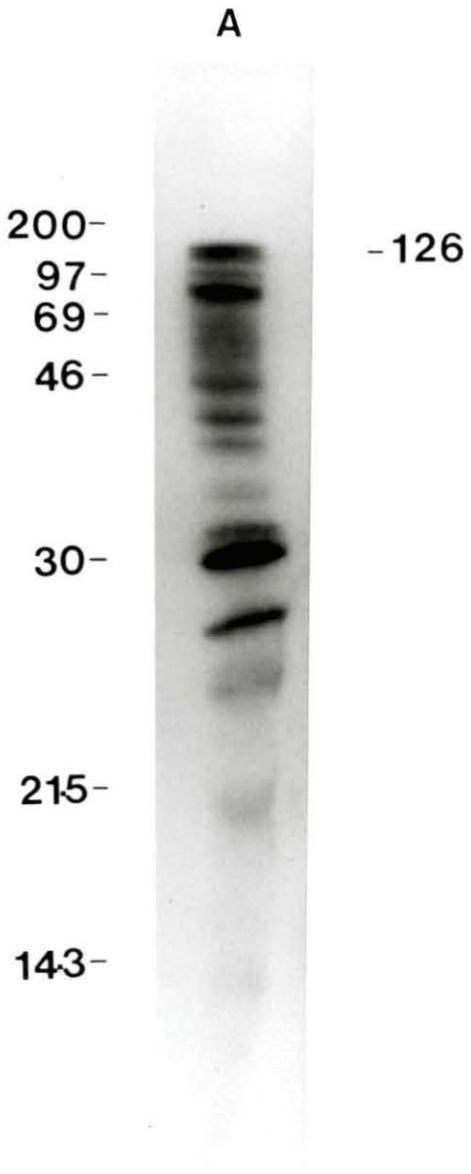

A
200-
97-
69-
46-
30-
215-
143-
-126

Figure 2. Western blot analysis of *M. dispar* reacted with antibodies against CBP. The antigens were whole cell proteins (840 μg protein/lane) of *M. dispar*. Dilutions of the antibodies from lane A to lane G were 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640 (1:10 representing 0.7 mg/ml globulins), respectively. Standard molecular weights are shown on the left side of the blot. Arrow indicates 126 kDa band.

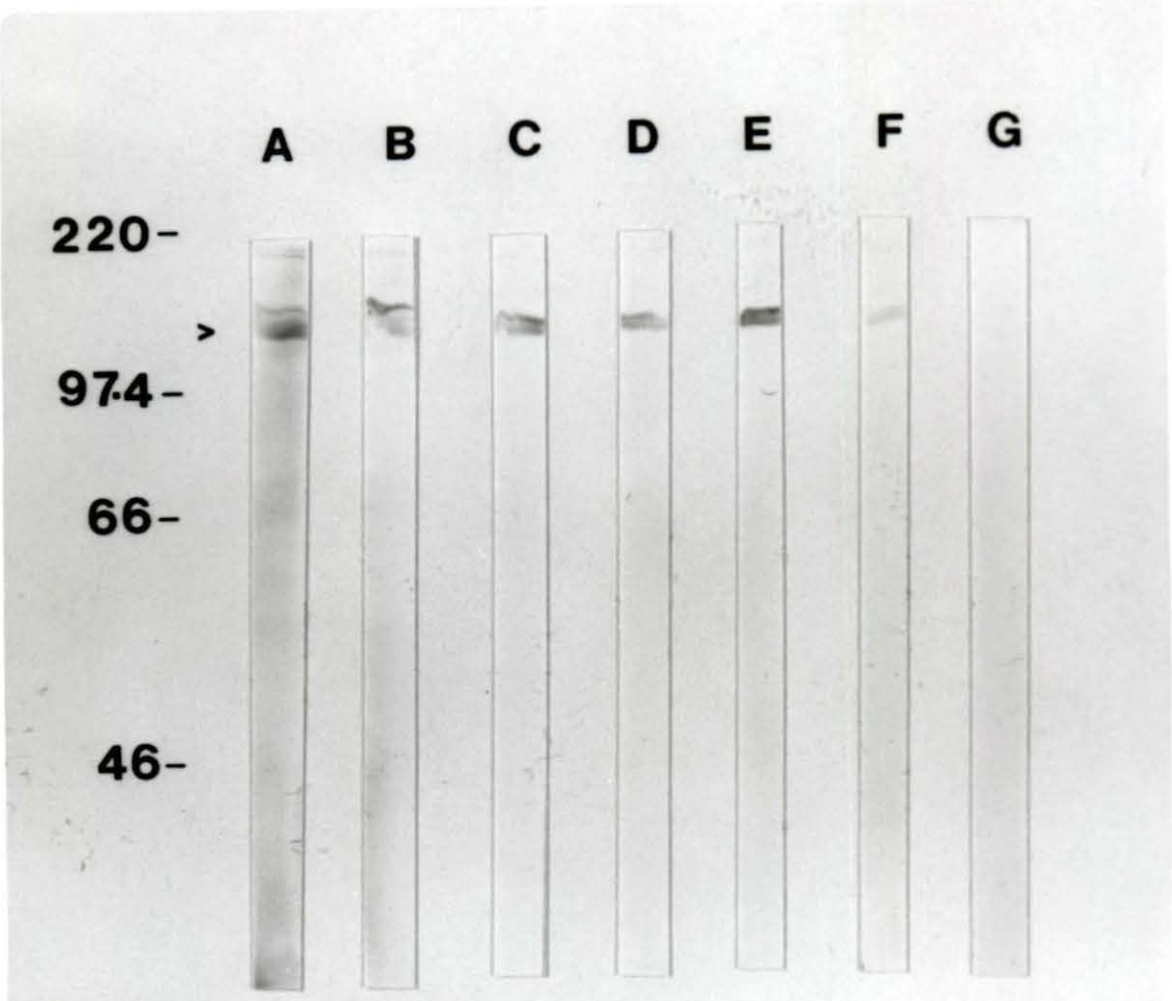

A
B
C
D
E
F
G
220-
97.4-
66-
46-

Figure 3. Western blot analysis of purified CBP reacted with antibodies against CBP. The antigen (purified from 840 μg cells/lane) of lane A and lane B was purified CBP. The antigen of lane C was capsule-coated RCA_{120} beads. Antibody dilution was 1:20 (0.35 mg/ml globulins) in lane A, 1:40 in lane B, and 1:20 in lane C. Standard molecular weights are shown on the left side of the blot. Arrow indicates 126 kDa band.

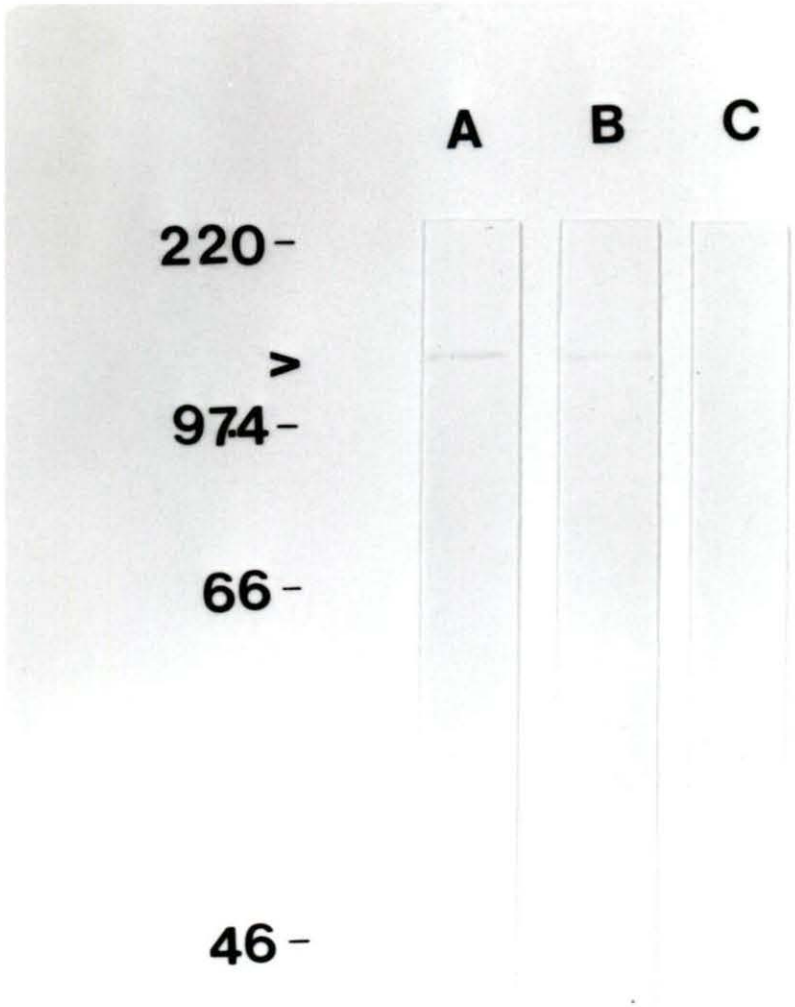

A
B
C
220-
>
97.4-
66-
46-

Figure 4. Western blot analysis of TX-114 phase partitioned *M. dispar* proteins with antibody against CBP (partitions from 1120 μg cell proteins). Lane A, total *M. dispar* proteins; Lane B, TX-114 phase; Lane C, aqueous phase. Antibody dilution was 1:20 (0.35 mg/ml globulins). Standard molecular weights are shown on the left side of the blot. Arrow indicates 126 kDa band.

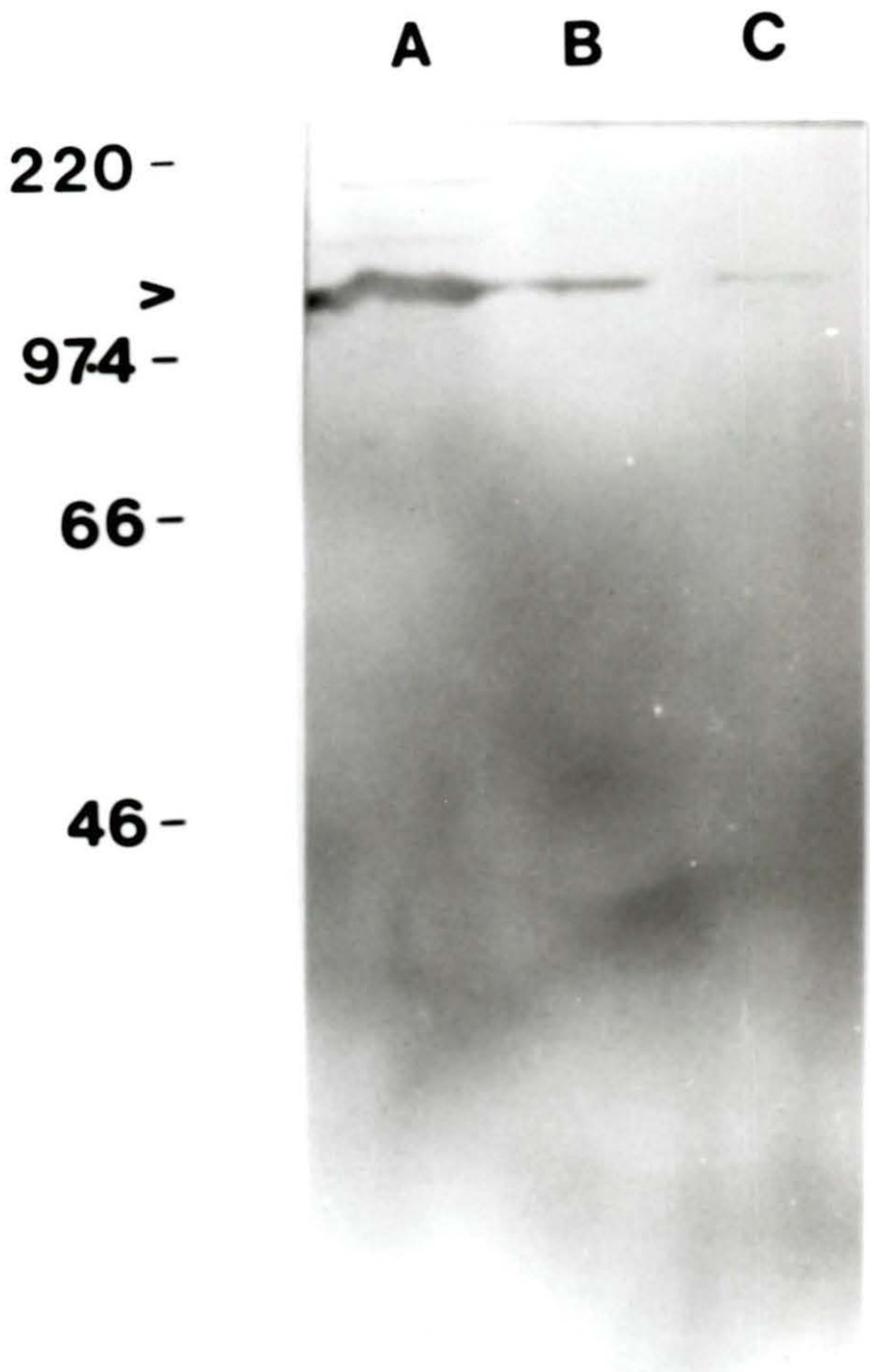

A
B
C
220
>
97.4
66
46

Figure 5. Western blot analysis of intact mycoplasma cells (equivalent to 1120 μg cell protein/lane) treated with TPCK-trypsin. Lane: A, intact cells treated with 10 μg/ml TPCK-trypsin; lane B, intact cells treated with 100 μg/ml TPCK-trypsin. Antibody dilution was 1:20 (0.35 mg/ml globulins). Arrows indicate the 126 kDa band and the smallest fragment.

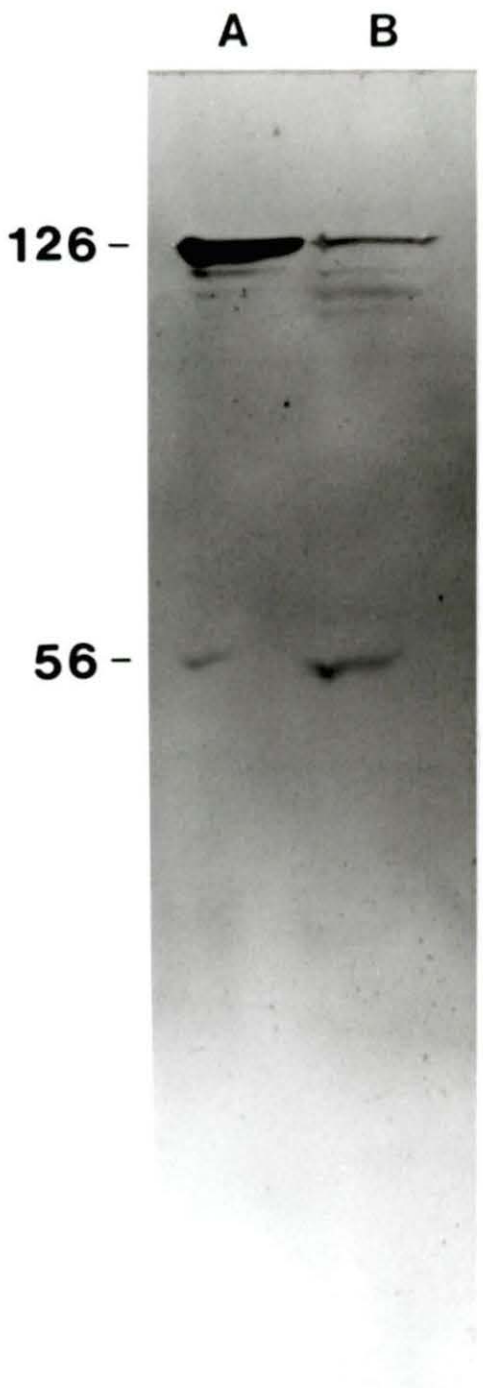

A
B
126
56

Figure 6. Western blot analysis of the effects of trypsin treatment of intact mycoplasma cells (1120 μg protein/lane) on the capsule-binding property of CBP. Lanes A-C, intact cells treated with TPCK-trypsin. Lane A, 1μg/ml TPCK-trypsin; lane B, 10 μg/ml TPCK-trypsin; lane C, 100 μg/ml TPCK. Lanes D-F, intact cells treated with TPCK-trypsin, then lysed, and the lysate reacted with capsule-coated RCA_{120} beads. Washed beads were loaded on gel. Lane D, CBP from cells treated with 1 μg/ml TPCK-trypsin; lane E, CBP from cells treated with 10 μg/ml TPCK-trypsin; lane F, CBP from cells treated with 100 μg/ml TPCK-trypsin. Antibody dilution was 1:20 (0.35 mg/ml globulins). Standard molecular weights are shown on the left side of the blot. Arrow indicates the 126 kDa band.

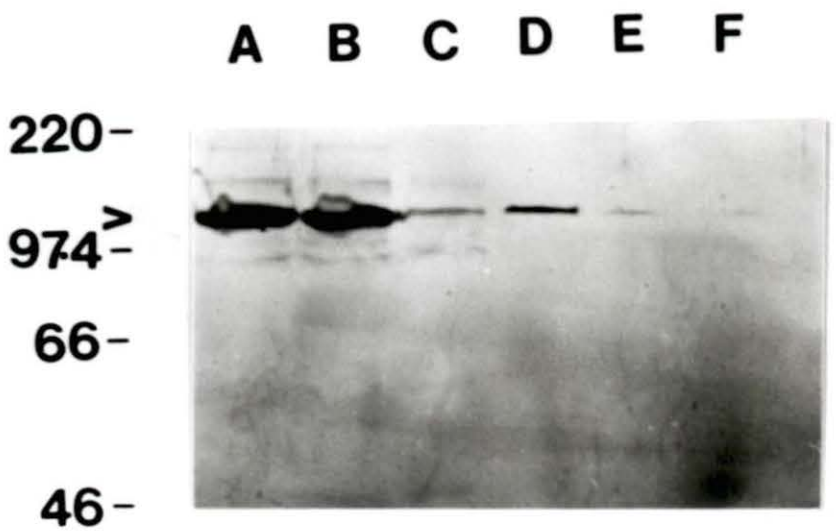

A
B
C
D
E
F
220-
97.4-
66-
46-

Figure 7. Western blot analysis of capsule-binding fragments released into supernatant after TPCK-trypsin- treatment of mycoplasma cells . Lane A, total supernatant from TPCK-trypsin-treated cells (total cell protein 28 mg); lane B, capsule binding protein from supernatant. Antibody dilution was 1:20 (0.35 mg/ml globulins). The molecular weight of the largest and the smallest fragments are indicated.

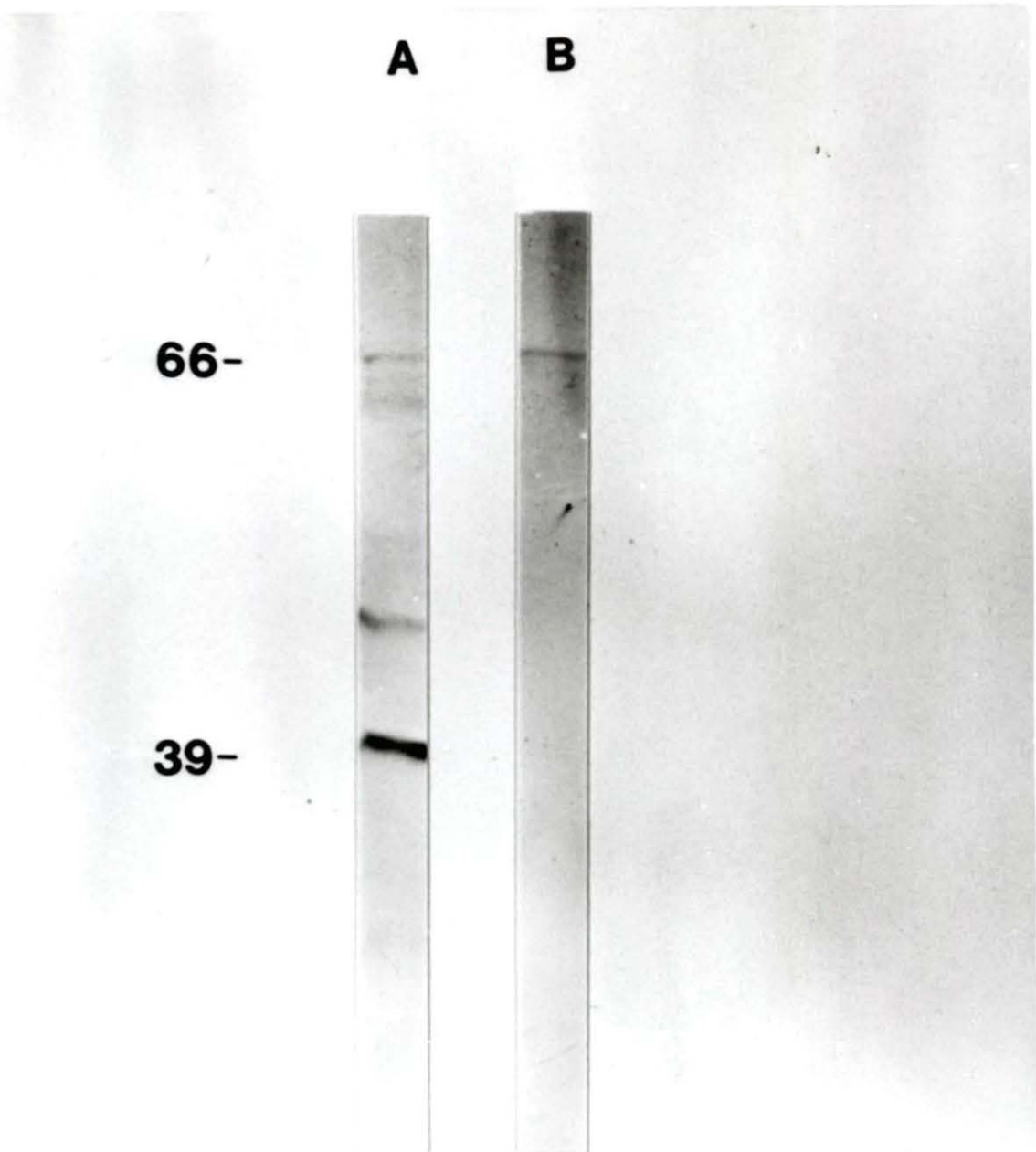
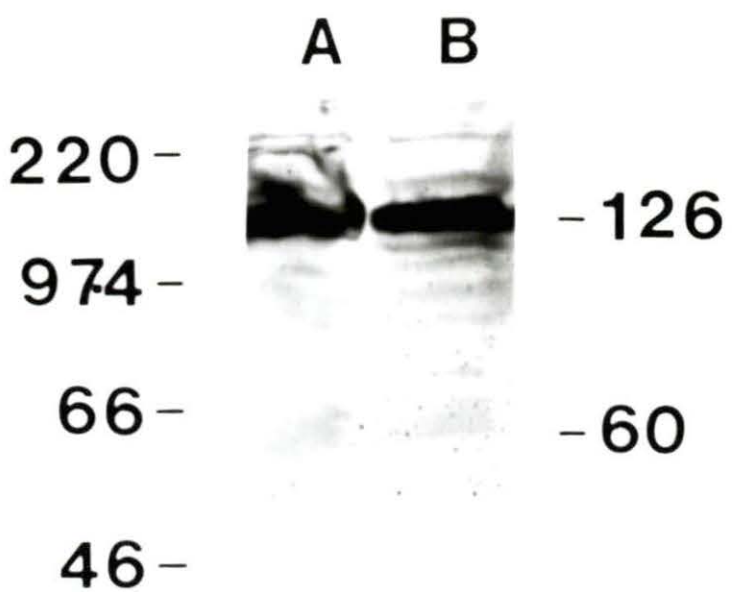

A
B
66-
39-

Figure 8. Carboxypeptidase Y analysis of CBP orientation on *M. dispar* cell membrane by Western blot with antibody against CBP (total protein 2240 µg);. Lane A, intact mycoplasma cells; lane B, mycoplasma cells treated with carboxypeptidase Y. Antibody dilution was 1:20 (0.35 mg/ml globulins). The 126 kDa protein and the smallest fragment were indicated on the right side. Standard molecular weights are shown on the left side of the blot.


A
B
220
97.4
66
46
126
60

DISCUSSION

Mycoplasma dispar can produce capsular polysaccharide, which exerts inhibitory effects on host immune response. CBP was found to bind the capsule and to be co-regulated with its production, and may therefore be associated with capsule production. Glucosyltransferases (GTFs) of mutans streptococci have been demonstrated to have glucan-binding and catalytic domains and be very important virulence factors (53, 93, 94, 109). *M. dispar* CBP may share some biological and structural properties with GTFs. This study used TX-114 detergent-phase fractionation to demonstrate hydrophobic domains within CBP, thereby categorizing CBP as an integral membrane protein. It further established that CBP is oriented with the C-terminus exposed externally to the single bilayer membrane of the organism and the capsule-binding domain is located in a 66 kDa tryptic fragment which appears to be at or near the C-terminal end.

To determine if CBP is an integral membrane protein in this study, a combination of polyclonal serum against CBP and application of TX-114 phase partitioning system was used. Membrane proteins are usually divided in two categories: integral proteins and peripheral proteins (132). The majority, integral proteins, are intercalated more or less deeply into or even through the lipid double layer and are held in position by strong, mostly hydrophobic interactions between hydrophobic amino acid side chains and lipid hydrocarbon chains. For identification of integral membrane proteins the Triton X-114 phase fractionation method has been suggested by some authors (13, 16, 114). Detergent phase partitioning has gained wide acceptance in selectively fractionating proteins

associated with a number of membrane systems (108). Two particular advantages, however, underscore the unique applicability of this technique to the study of mycoplasma membrane proteins. First, unlike eubacteria and eucaryotes, mycoplasmas have only a single plasma membrane system which is essentially responsible for all communication of these organisms with their external environment. As a result, proteins identified during phase partitioning by their intrinsic property of hydrophobicity are selected as components likely to be associated with this single membrane system. It is therefore an efficient method to focus on a group of mycoplasma components having probable roles in important membrane functions. Second, it is generally established that with the notable exception of acholeplasma, most mycoplasmas yield membrane preparations by standard disruption procedures that are significantly contaminated with intracellular and extracellular macromolecules (110). The use of TX-114 phase partitioning is perhaps ideally suited, therefore, for the segregation of mycoplasma membrane proteins from these contaminants. TX-114 phase partitioning of CBP resulted in most of the CBP being found in the TX-114 phase. This identified that the CBP has hydrophobic domains and defined CBP as an integral membrane protein. For the integral membrane proteins, there is a range of hydrophobicities, from those having only one putative hydrophobic transmembrane segment to those having several to many segments, in relation to the lengths of the proteins and their hydrophilic parts. Since some CBP was also found in the aqueous phase, further analysis will need to be done to understand the structure of CBP.

Because of the possibility raised by this study that the CBP could be a surface

exposed protein, further delineation of the orientation and molecular location was sought. Trypsin (which cleaves predominantly at Lys and Arg residues) has been used extensively in many systems to demonstrate surface proteins on intact cells (61, 114, 121). The CBP was established to be exposed at the mycoplasma surface, since it was susceptible to the action of trypsin on intact organisms, which removed all capsule-binding cleavage fragments from membrane association.

In addition to the 126 kDa band, there were two other bands >126 kDa shown in Fig. 2, 4, 6, 8. The possible reason for why the antibodies against CBP also reacted with other large proteins is that when we made CBP antigens, some degraded 126 kDa fragment from the large proteins was also contained in the gel slices. In the TX-114 partitioning experiment, the proteins larger than 126 kDa did not appear in the TX-114 phase. This would indicate these proteins are not membrane related. Nonspecific reactivity of antibodies against CBP may also account for results. For instance, the 39 kDa fragment produced a very strong band compared to others. One possible reason is that several fragments of similar size were generated from more than one protein species.

Large proteins are organized in domains (66), in part because this aids protein three-dimensional assembly during protein synthesis. Protein folding is more manageable when it occurs in discrete segments, which minimizes thermodynamic and steric complexities of assembling a large random structure (181). There are evolutionary advantages to domain structure as well. Protein segments which can maintain native form and function independently of external stabilizing forces have fewer structural demands when incorporated into a new

protein through recombination (66). Therefore, the CBP of *M. dispar* is supposed to have structural domains. Intact CBP has the capacity to bind *M. dispar* capsule. This suggests that *M. dispar* CBP has capsule-binding domains. Mild trypsin digestion of intact *M. dispar* cells generates several large fragments that are associated with the mycoplasma cells, but none of these fragments have the capacity to bind *M. dispar* capsule. A single 66 kDa fragment from the supernatant displays capsule-binding function equivalent to that of the intact CBP. This fragment appears to contain the full size of the capsule binding domain. Kobayashi et al. (81) reported a 55-kDa glucan-binding fragment from GTF-S and GTF-I of *Streptococcus sobrinus* after proteolysis, and Mooser and Wong reported a 60-kDa glucan-binding fragment. Size similarity among these fragments may underscore other similarities. The binding domain of *M. dispar* CBP may have similar repeating structures as those of the GTFs of mutans streptococci. To confirm this, amino acid sequence analysis of the 66-kDa fragment will be needed.

Integral membrane proteins can be divided into four classes according to their topology in the membrane (174, 175). Class I proteins have a cleavable N-terminal signal peptide followed by a hydrophilic loop and a hydrophobic anchor sequence. The latter is followed by a cluster of positively charged amino acids, and the final orientation of processed proteins is N terminus out/C terminus in. Class II proteins have an uncleaved signal peptide acting as an anchor, giving the proteins an N terminus in/C terminus out topology. Class III proteins are similar to Class II proteins, but have an N terminus out/C terminus in orientation due to presence of positive clusters after the anchor segment. Class IV proteins

have multiple hydrophobic regions spanning the membrane. The *M. dispar* CBP appears to belong to Class II, because it was demonstrated that CBP had a N terminus in/C terminus out topology. Carboxypeptidase Y can successively cleave C-terminal amino acid residues and hydrolyses Lys, Arg, and His residues at greatly reduced relative rates (58). Digestion of intact *M. dispar* cells with carboxypeptidase Y yielded a ladder with a minimum size of 60 kDa. The spacing of the products was the same. This result suggested that the C-terminal portion of CBP is accessible at the external surface of the organism. In addition, it also suggested that CBP is not anchored in the membrane by C-terminal regions, but rather is anchored by the N-terminal portion of the molecule. In the result, the generated fragment bands were weak and most of the CBPs were not digested. One possible reason may be that the capsule-releasing step did not release most of the capsules. Therefore, capsule protected the CBP from being digested.

We have shown that *M. dispar* CBP is an integral membrane protein with its N-terminal portion anchored in the membrane and C-terminus external and its capsule-binding domain is located at or near the C-terminal region. Detailed structural and functional analysis of *M. dispar* CBP with emphasis on its role in capsule production and its interaction with the capsule will give insights into the understanding of capsule synthesis mechanism and may lead to the development of a vaccine to control the disease caused by *M. dispar*. The results presented here also provide the necessary basis for further studies of *M. dispar* CBP.

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