INFECTION AND IMMUNITY, Feb. 1996, p. 413–421 Vol. 64, No. 2
0019-9567/96/$04.00+0
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An Oxygen-Induced but Protein F-Independent Fibronectin-Binding Pathway in *Streptococcus pyogenes*

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Received 28 August 1995/Returned for modification 12 October 1995/Accepted 8 November 1995

Protein F is an important fibronectin-binding adhesin of *Streptococcus pyogenes* (group A streptococcus). However, all previous analyses of protein F have been conducted in a mutant strain which expresses protein F under anaerobic conditions nonpermissive for expression in other strains. In this study, we have examined the fibronectin-binding properties of several protein F-deficient mutants cultured under aerobic conditions and have identified a second pathway for binding fibronectin. Unlike the case with protein F, exposure to an aerobic environment does not induce transcription of a new gene product. Rather, O_2 is apparently required for the modification of a protease-resistant cell surface component into a binding-competent form. Modification occurred preferentially at a pH of 6.0 or less, and the binding of the modified component to fibronectin required Zn^{2+}. The oxidizing agent $Fe(CN)_6$ could be substituted for O_2 and stimulated expression of binding activity under O_2-limiting conditions. Streptococcal fibronectin binding mediated by this pathway but not by protein F could be inhibited by laminin and by streptococcal lipoteichoic acid, a molecule previously implicated as the streptococcal adhesin for fibronectin. The non-protein F-binding activity could also substantially enhance the binding of the organism to basement membrane. By using differential inhibition, analyses of binding to non-protein F mutant strains demonstrated that the total level of fibronectin bound under aerobic conditions reflects contributions from both pathways. Because of its dependence on Zn^{2+}, an oxidant, and pH, this binding activity has been designated the ZOP binding pathway.

The highly refined and regulated interplay between an infecting microorganism and its host typically begins with the recognition of a specific host cell structure (referred to as a receptor) by a highly evolved adhesive molecule ("adhesin") of the microorganism (20). Considerable evidence has accrued to suggest that recognition of the eukaryotic protein fibronectin contributes to the ability of many different microbial species to interact with various host tissues and cells during the course of infection (9, 29). Fibronectin can be found in a soluble form in most body fluids or as an immobilized form in association with cell surfaces or as a component of the extracellular matrix (21). An interesting feature of fibronectin is its ability to bind to a large heterogeneous population of substrates, including integrins, collagens, fibrin, DNA, heparin, and other protein and nonprotein compounds (21). This wide spectrum of binding activities combined with its broad distribution within the host has made fibronectin an attractive target as a microbial receptor.

Fibronectin binding has been extensively studied in the gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus). This microorganism is the etiologic agent of numerous suppurative infections of the pharynx (e.g., strep throat) and soft tissues (impetigo and necrotizing faciitis) as well as several systemic diseases that can result from toxigenic (scarlet fever and toxic shock-like syndrome) or immunopathological (rheumatic fever) processes. *S. pyogenes* was one of the first bacterial species for which the ability to bind fibronectin was demonstrated (25, 40), and evidence has rapidly accumulated to suggest that binding to fibronectin is a strategy used by *S. pyogenes* to adhere to certain host tissues during infection (reviewed in references 1, 17, and 37).

Because of this intense interest, it is not surprising that a large body of conflicting data has been generated concerning the mechanism(s) by which *S. pyogenes* recognizes and binds to fibronectin. Both lipoteichoic acid (LTA) and numerous different proteins have all been implicated as the streptococcal adhesin for fibronectin. LTA is a prominent component of the streptococcal cell surface, and it has been proposed to be the streptococcal fibronectin-binding adhesin primarily on the basis of the ability of purified LTA to inhibit the binding of fibronectin to intact streptococcal cells (1, 17, 37). LTA is an amphipathic linear polymer of glycerol phosphate, linked by 1-3 phosphodiester bonds, which contains a terminal glycerophosphoryl diglucosyl diglyceride lipid moiety (47). It is this terminal lipid domain of LTA that binds to fibronectin (1, 37); however, since this domain anchors LTA to the cell (47), it is not normally exposed on the cell surface in a conformation in which it could interact with fibronectin. It has been suggested that LTA can form a complex with a surface protein, such as the M protein (27), that reorients LTA to expose its lipid domain on the cell surface (1, 27, 37). However, more recent data have demonstrated that expression of M protein is not essential for the ability to bind to fibronectin (4).

The proteins that have been implicated as fibronectin-binding adhesins include the 28-kDa antigen (6), FBP54 (7), glyceraldehyde-3-phosphate dehydrogenase (30), a serotype 3 M protein (33), serum opacity factor (31), and the closely related proteins sfb (41, 43) and protein F (14). Perhaps the best characterized of these are members of the sfb/protein F family, which contain two distinct domains for binding to fibronectin (36). One binding domain recognizes the N-terminal domain of the fibronectin molecule and consists of a 27-amino-acid motif that is repeated up to six times in tandem (26, 36, 42, 43). This domain is similar to the repetitive binding domains of the

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fibronectin-binding proteins of *Staphylococcus aureus* and *Streptococcus dysgalactiae* (23). The second binding domain of protein F is located immediately N-terminal to the repeat-binding domain and recognizes a region of fibronectin that is distinct from the region recognized by the repeat-binding domain (36). Both domains of protein F are required for optimal binding to fibronectin (36).

The observation that the introduction of the gene which encodes protein F (*prtF*) into nonbinding isolates of *Enterococcus faecalis* and *S. pyogenes* confers upon these strains the ability to bind fibronectin (16), coupled with the observation that insertional inactivation of *prtF* in one strain of *S. pyogenes* (JRS4) completely abolished its ability to bind fibronectin (14), have provided genetically based evidence to suggest that expression of protein F is essential for the ability of *S. pyogenes* to bind to fibronectin. However, in the latter example, it appears that JRS4 is a regulatory mutant which constitutively expresses protein F under all culture conditions (45), apparently as a result of a mutation in the regulatory gene *rofA* (12). All other strains of *S. pyogenes* regulate expression of protein F in response to elevated levels of oxygen and will not express this protein when cultured anaerobically (12, 45). All previous analyses of the binding characteristics of *prtF* mutants have been conducted in the JRS4 constitutive expression background under low-oxygen culture conditions that are normally nonpermissive for *prtF* expression in other strains (14).

In this study, we have analyzed the ability of *prtF* mutant strains to bind fibronectin when cultured aerobically and have identified a second pathway for binding fibronectin that is distinct from protein F. This pathway does not involve the induction of a new gene product in the presence of oxygen. Instead, oxygen is apparently required for the modification of a protease-resistant surface structure in a pH-sensitive reaction which permits fibronectin binding in a Zn^{2+}-dependent manner. Because of its dependence on Zn^{2+}, oxygen, and pH, this mechanism of binding has been designated the ZOP pathway. Furthermore, the observation that binding via the ZOP pathway can be inhibited by LTA (while binding via protein F is insensitive to LTA) suggests that ZOP binding may represent the binding activity previously attributed to LTA.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* DH5α (Bethesda Research Laboratories) and *Enterococcus faecalis* OG1X (22) were used in molecular cloning and fibronectin-binding experiments. *S. pyogenes* JRS4 (35) produces a type 6 M protein and is a spontaneous streptomycin-resistant derivative of strain D471 from the Rockefeller University collection. Insertional inactivation of *emm6.1* in JRS4 generated JRS145 (2). Insertional inactivation of *prtF* in JRS4 and JRS145 generated SAM1 and SAM2, respectively (14). *S. pyogenes* HSC5, which produces a type 5 M protein (16), is unrelated to JRS4.

Growth conditions. *E. coli* was cultured in Luria-Bertani broth (34) at 37°C with agitation. *S. pyogenes* strains were cultured in Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco) (THY medium). Solid medium was produced by adding Bacto agar (Difco) to THY medium to a final concentration of 1.4%. Unless stated otherwise, *S. pyogenes* strains grown in liquid medium were incubated overnight at 37°C without agitation in sealed culture bottles. Where indicated, liquid cultures were supplemented with $K_3Fe(CN)_6$ (Sigma) to a final concentration of 1 mM (45). *S. pyogenes* cultured on solid medium were incubated in ambient air. In selected experiments, *S. pyogenes* was cultured in chemically defined medium (CDM) (44).

Construction of HSC10. *prtF* in oxygen-regulating strain HSC5 (45) was inactivated by a strategy identical to that used to construct SAM1 (14) and is summarized as follows. Plasmid pPTF6.1 contains the *prtF1*::ΩKm-2 null allele of *prtF* that was constructed by inserting the ΩKm-2 element into the *prtF* coding region (14). Digestion of pPTF6.1 with *Eco*RI converted the plasmid to a linear molecule that was then used to transform HSC5 by electroporation (3, 15), with selection for the kanamycin resistance determinant of ΩKm-2. Transformants resistant to kanamycin (500 μg/ml) arose from homologous recombination between the *prtF* sequences shared by the chromosomal allele and *prtF1*::ΩKm-2 that resulted in the introduction of ΩKm-2 into the chromosome and the replacement of the resident allele with *prtF1*::ΩKm-2. Replacement in one transformant (designated HSC10) was confirmed by a Southern blot analysis with the appropriate DNA probes, and the failure to express protein F was confirmed by a protein transfer blot of surface proteins prepared from HSC10 probed with ^{125}I-fibronectin by the method described previously (14). Since *prtF1*::ΩKm-2 is stably maintained in the absence of selection, media for routine culture of HSC10 and SAM1 for fibronectin-binding assays did not include kanamycin.

Analysis of fibronectin-binding activity. Bacteria cultured as described above or treated under the various conditions described in the text were harvested by centrifugation, washed twice in an equal volume of phosphate-buffered saline (PBS, pH 7.4) containing 1% (vol/vol) Tween 20 (PBS-T) and then resuspended in PBS-T to an optical density at 600 nm (OD_{600}) of 0.2. The ability of the bacteria to bind ^{125}I-fibronectin was then determined as described previously (14, 16). Under these conditions, positive control strains typically bound 25,000 to 35,000 cpm of ^{125}I-fibronectin, while the negative control *E. coli* DH5α bound 2,500 cpm or less. In inhibition experiments, the potential inhibitors described in the text (purified peptidoglycan was the gift of William Goldman, Washington University; all other inhibitors were obtained from Sigma) were incubated with *S. pyogenes* for 2 h at room temperature prior to incubation with ^{125}I-fibronectin. In other experiments, the various detergents described in the text (sodium deoxycholate was from Calbiochem; all others were obtained from Sigma) were added to the bacterial suspensions to a final concentration of 0.1% and incubated at room temperature for 30 min prior to incubation with ^{125}I-fibronectin. The percent inhibition of binding in the presence of each detergent was calculated as follows: [1 − (cpm of ^{125}I-fibronectin bound in the presence of detergent in PBS/cpm of ^{125}I-fibronectin bound in PBS-T)] × 100. The lysate of *E. coli* used as an inhibitor was prepared, and the inhibition analysis was conducted as has been described in detail elsewhere (14). The data presented represent the mean derived from a minimum of three independent experiments, and each individual experiment was conducted in duplicate. The data from any individual experiment were rejected if the values from each duplicate differed by more than 5%.

Evaluation of reaction conditions. SAM1 cells from a 100-ml liquid culture in THYB were harvested by centrifugation and resuspended in 1.0 ml of PBS. A 100-μl aliquot of this suspension was then added to 10 ml of the various reaction media supplemented as described in the text in the presence or absence of 1.0 mM $Fe(CN)_6$. Following incubation at 37°C for 1 h, the cells were harvested by centrifugation and resuspended in PBS-T (pH 7.4), and their ability to bind ^{125}I-fibronectin was determined as described above. All reagents used were obtained from Sigma except for desferoxamine mesylate (CIBA-Geigy) and 1,10-phenanthroline monohydrate (Fisher). Ultrafiltration of media was done with microconcentrator cartridges (Centricon) according to the directions of the manufacturer (Amicon). Where indicated, cells were treated with trypsin as follows. Cells from a 100-ml culture were harvested and resuspended as described above in 10 ml of PBS (pH 7.4), to which 500 μg of typsin (Sigma) was then added. Following a 1-h incubation at 37°C, 500 μg of soy bean trypsin inhibitor (Sigma) was added, the cells were harvested by centrifugation and washed twice in PBS-T, and their ability to bind ^{125}I-fibronectin was determined. In selected experiments, the ability of heat-inactivated bacteria to bind fibronectin was determined following exposure of an overnight culture to a temperature of 121°C for 15 min in an autoclave at 15 lb/in^2. In other experiments, the surface hydrophobicity of treated cells was measured by the method of Rosenberg et al. (32).

Adherence to basement membrane. Solubilized basement membrane purified from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma (Matrigel, Collaborative Biomedical) was diluted to 4.3 mg/ml in minimal essential medium (MEM), and 40-μl aliquots were used to coat the surfaces of glass coverslips (diameter, 1 cm) that were placed in the wells of a 24-well tissue culture plate. Following a 1-h incubation at room temperature to allow the membrane to gel, 500 μl of blocking buffer (PBS, 0.2% bovine serum albumin [BSA], 0.5% Tween-20) was added to each well and allowed to incubate for 30 min at room temperature. The buffer was removed by aspiration and replaced with 500 μl of a bacterial suspension adjusted to an OD_{600} of 0.150 in PBS-T. After a 2-h incubation at room temperature, each well was washed five times in PBS-T, and adherent streptococci were visualized by staining with acridine orange and fluorescent microscopy as described previously (28).

RESULTS

Analysis of mutants reveals a protein F-independent fibronectin-binding activity. It has been shown that transcription of the gene which encodes protein F (*prtF*) is regulated in response to oxygen (12, 45). However, *prtF* is constitutively expressed in JRS4, apparently as a result of a mutation in *rofA*, a gene which positively regulates transcription of *prtF* (12). These binding phenotypes are reviewed in Fig. 1, which illustrates that JRS4 has a constitutive binding phenotype and binds fibronectin when cultured in liquid THY medium (an oxygen-limited environment) (45) and on the surface of solid THY medium (an aerobic environment) (45) (Fig. 1A). In contrast, HSC5 demonstrates the regulated binding phenotype

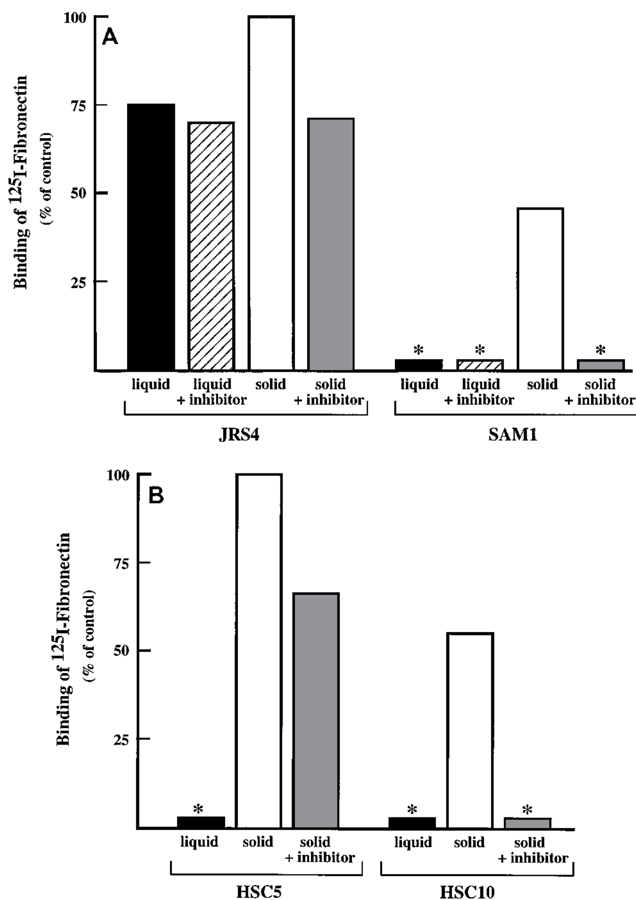

FIG. 1. Analysis of mutants reveals a second pathway for binding fibronectin. The abilities of *S. pyogenes* JRS4 and isogenic protein F-deficient mutant SAM1 (A) and HSC5 and isogenic protein F-deficient mutant HSC10 (B) to bind to fibronectin following culture in liquid THY medium (an O_2-limited environment) (45) or on solid THY medium (an aerobic environment) (45) are shown. The ability of the strains to bind fibronectin in the presence of a sonic lysate of *E. coli* (inhibitor) is also shown. Binding is quantitated relative to the binding of JRS4 (A) or HSC5 (B) cultured aerobically. *, binding higher than background values was not observed. The data reported represent mean values from a minimum of three independent experiments, which differed by less than 5%.

and will only bind fibronectin following culture on solid THY medium (Fig. 1B) (45).

Since the previous studies which indicated an essential role for protein F in fibronectin binding had been conducted in the JRS4 host background under the oxygen-limited conditions nonpermissive for *prtF* expression in HSC5 and other strains of *S. pyogenes* (14), it was of interest to analyze the fibronectin-binding properties of *prtF* mutants following aerobic culture. As shown previously (14), a strain derived from JRS4 by insertional inactivation of *prtF* lost its ability to bind fibronectin following liquid culture (SAM1 [Fig. 1A]). Surprisingly, this *prtF*-deficient mutant was capable of binding fibronectin when tested following culture under aerobic conditions, although its binding was approximately 50% lower than that of JRS4 under the conditions of this assay (SAM1 [Fig. 1A]). Similarly, insertional inactivation of *prtF* in regulating strain HSC5 generated a strain (HSC10) whose ability to bind fibronectin following culture on solid medium was also reduced but not eliminated (Fig. 1B). As with protein F (14), subsequent incubation with a large excess of unlabeled fibronectin (100-fold) for an extended time period (18 h) did not result in the displacement of labeled fibronectin from the streptococcal cells (not shown). *E. faecalis* OG1X did not bind detectable amounts of fibronectin following culture on either liquid or solid THY medium (not shown). These data suggest that following aerobic culture, *S. pyogenes* can express a second fibronectin-binding activity in addition to protein F. This second activity does not require expression of the M protein, since the results obtained with an M^- derivative of JRS4 (JRS145) were identical to those with JRS4, and the results obtained with an F^- derivative of JRS145 (SAM2) were identical to those with F^- M^+ strain SAM1 (not shown).

Because cloned surface proteins of gram-positive bacteria are not expressed on the surface of *E. coli*, protein F was cloned by a strategy of testing whole cell lysates of an *S. pyogenes* gene library prepared in *E. coli* DH5α for a cloned activity that would compete for fibronectin binding with whole cells of JRS4 (14). This assay was successful, because the *E. coli* whole-cell lysate itself does not inhibit fibronectin binding to protein F (14) (compare JRS4 columns "liquid" with "liquid + inhibitor" in Fig. 1A). However, a very different result was obtained in attempts to clone the non-protein F fibronectin-binding activity by the same strategy, in that the *E. coli* lysate itself was an effective inhibitor of the binding of fibronectin to the non-protein F-binding activity (Fig. 1, compare columns "solid" with "solid + inhibitor" for both SAM1 and HSC10). The observation that the lysate could partially inhibit the binding of the parental strains grown on solid medium (JRS4 and HSC5 in Fig. 1A and B) provides additional evidence that these strains express two fibronectin-binding activities; a protein F activity which is not inhibited by the lysate, and a second activity that is inhibited by the lysate.

An oxidant can promote expression of the non-protein F binding activity in liquid medium. While the *E. coli* lysate assay was not useful for cloning the non-protein F binding activity, it did provide a useful method for discrimination between protein F- and non-protein F-directed fibronectin binding for further comparison of these two pathways. In a previous study, it was shown that the addition of the oxidizing agent $Fe(CN)_6$ to liquid culture provided an effective stimulant for the expression of fibronectin-binding activity (45) (see also HSC5 in Fig. 2). This increase in binding activity had been attributed to the induction of protein F; however, the binding stimulated by $Fe(CN)_6$ can be completely inhibited by the *E. coli* lysate (HSC5 in Fig. 2). In addition, $Fe(CN)_6$ also stimulated a lysate-inhibitable binding in *prtF* mutants SAM1 and HSC10 (Fig. 2). Neither *E. coli* DH5α nor *E. faecalis* OG1X bound detectable amounts of fibronectin following culture with $Fe(CN)_6$ (not shown). In JRS4, the addition of $Fe(CN)_6$ slightly increased the already high levels of binding, and addition of the lysate only inhibited binding to the levels obtained in the absence of the oxidant (Fig. 2). These data suggest that $Fe(CN)_6$ does not stimulate the expression of protein F, as previously thought, but rather stimulates expression of the protein F-independent binding activity.

Laminin and LTA inhibit fibronectin binding by the non-protein F binding activity. From the data presented above and previous mutagenesis studies (14), it appears that all fibronectin-binding activity of JRS4 following culture in liquid medium occurs exclusively via protein F. In contrast, when *prtF* mutant SAM1 is cultured in liquid medium supplemented with $Fe(CN)_6$, all binding occurs via the non-protein F activity. This ability to generate conditions which result in the expression of only one or the other of the two activities was used to examine the abilities of various substances to inhibit the binding of fibronectin by each of the binding pathways. As expected, the addition of an excess of unlabeled fibronectin to the binding

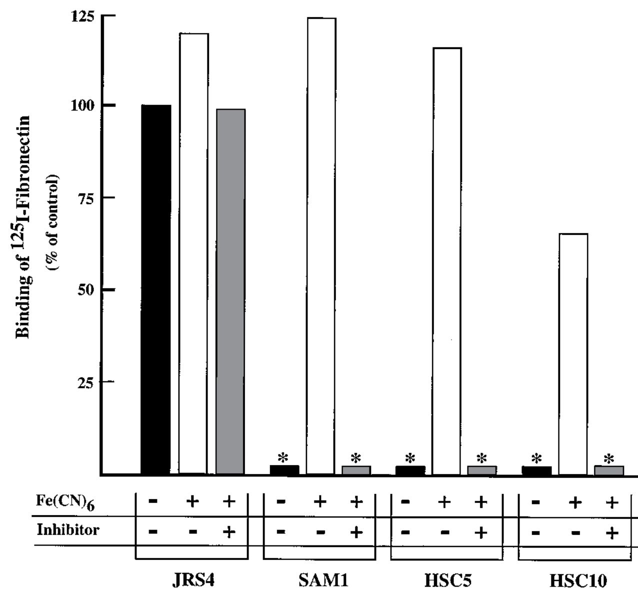

FIG. 2. An oxidant can stimulate non-protein F binding activity in anaerobic culture. The indicated strains were cultured in liquid THY medium in the presence (+) or absence (−) of 1 mM $Fe(CN)_6$, and their abilities to bind fibronectin were evaluated in the presence (+) and absence (−) of a sonic lysate of *E. coli* (inhibitor). Binding is quantitated relative to the binding of JRS4 cultured anaerobically. *, binding higher than background values was not observed. The data reported represent mean values from a minimum of three independent experiments, which differed by less than 5%.

TABLE 1. Effects of various detergents on fibronectin binding

Detergent[a]	% Inhibition of binding[b] to: SAM1	JRS4
Triton X-100	14.0	21.2
IGEPAL CA-630	13.4	22.4
Nonidet P-40	8.9	22.9
SDS	100	92.0
Sarcosyl	100	26.8
Deoxycholate	28.5	+14.6

[a] The final concentration of all detergents was 0.1%.
[b] Inhibition of fibronectin binding relative to binding with 1.0% Tween 20 was calculated as described in Materials and Methods. Data represent means of duplicate determinations, which differed by less than 5.0%. SAM1 was cultured in THYB plus 1 mM $Fe(CN)_6$ to promote expression of the non-protein F fibronectin activity. JRS4 was cultured in THYB with no $Fe(CN)_6$ to ensure that all binding activity was generated by protein F.

assays effectively inhibited the binding of ^{125}I-fibronectin to both JRS4 cultured in liquid THY medium and SAM1 cultured in liquid medium with $Fe(CN)_6$ (Fig. 3). Neither BSA, lysozyme, nor gelatin had an appreciable inhibitory effect on binding via either of the two pathways (Fig. 3) and lipopolysaccharide, DNA, sialic acid, and peptidoglycan also did not inhibit binding to either pathway (not shown). However, while the matrix protein laminin did not inhibit binding to protein F, laminin could effectively inhibit fibronectin binding by the other activity (Fig. 3). Similarly, LTA could inhibit the binding of fibronectin by the non-protein F activity, while it had no ability to inhibit binding by protein F (Fig. 3). Different spectrums of inhibition by various detergents were also observed. While several nonionic detergents (Triton X-100, IGEPAL CA-630, and Nonidet P-40) had only moderate abilities and the ionic detergent sodium dodecyl sulfate (SDS) had considerable ability to inhibit the binding of fibronectin via either pathway, the ionic detergent Sarcosyl was a much stronger inhibitor of binding by the non-protein F pathway than by protein F (Table 1). Deoxycholate had an intermediate ability to inhibit binding by the non-protein F pathway and actually enhanced fibronectin binding by protein F (Table 1).

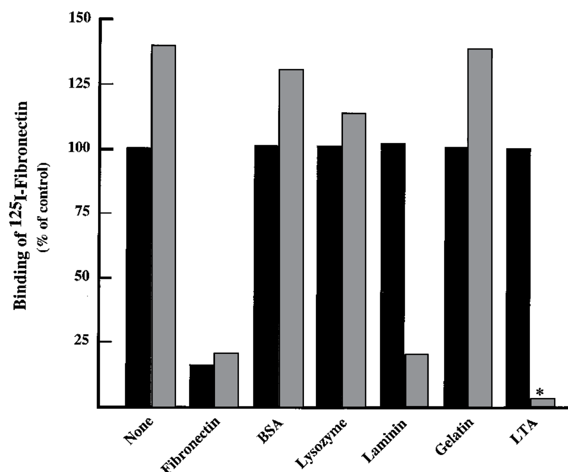

FIG. 3. Abilities of various substances to inhibit fibronectin binding to each binding pathway. JRS4 cultured in liquid THY medium (solid bars) will exclusively express protein F, while isogenic protein F-deficient mutant SAM1 cultured anaerobically in the presence of 1 mM $Fe(CN)_6$ (shaded bars) will exclusively express the non-protein F binding pathway. The ability of 1 μg of various substances to inhibit binding to each pathway is shown. Binding is quantitated relative to the binding of JRS4 cultured anaerobically. *, binding higher than background values was not observed. The data reported represent mean values from a minimum of three independent experiments, which differed by less than 5%.

Expression of the non-protein F binding activity does not require de novo protein synthesis. To examine the kinetics of induction of the non-protein F activity in the presence of $Fe(CN)_6$, SAM1 from an overnight liquid culture grown in the absence of $Fe(CN)_6$ was used to inoculate a liquid culture that contained $Fe(CN)_6$ (1.0 mM). Samples were removed at various time points and examined for their fibronectin-binding activities (Fig. 4). This analysis revealed that the appearance of binding activity paralleled the growth of the culture and was maximal in stationary-phase cells (Fig. 4). However, cell growth was not essential for expression, since $Fe(CN)_6$ could be added to cells grown to stationary phase in the absence of $Fe(CN)_6$, and following a 1-h incubation at 37°C, these cells exhibited binding at levels equivalent to those of cells grown to stationary phase in the presence of $Fe(CN)_6$ (not shown). Furthermore, de novo protein synthesis did not appear to be required for the expression of binding activity, because the addition of 40 μg of chloramphenicol per ml to stationary-phase cells had no effect on the amount of binding activity generated following a 1-h incubation with $Fe(CN)_6$. This concentration of chloramphenicol is approximately 50 times higher than the MIC for this strain. Consistent with the results obtained with chloramphenicol, inactivation of cell viability by heating also did not impair the generation of binding activity following incubation with $Fe(CN)_6$ (not shown).

Evaluation of reaction conditions. The results in the previous section suggested that the expression of binding activity in the presence of an oxidant did not require the induction of any new gene products or synthesis of new polypeptides but rather occurred as the result of the modification of an existing molecule. The conditions which promote this modification were

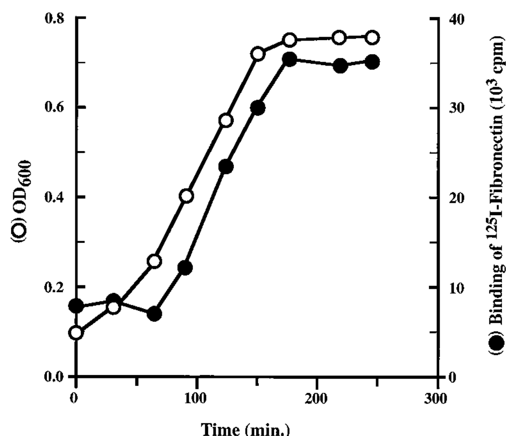

FIG. 4. Development of binding activity during culture. The growth of SAM1 cultured in liquid THY medium in the presence of 1 mM $Fe(CN)_6$ is shown on the axis on the left of the figure, while the ability of cells harvested at various time points to bind fibronectin is shown on the axis on the right of the figure. Binding was quantitated with each sample adjusted to an equivalent number of cells, as determined by the OD_{600}. Data represent the mean of a duplicate determination at each time point, each of which differed by less than 5%.

then examined in greater detail. Unexpectedly, binding activity was not generated if streptococci from an overnight liquid culture in the absence of $Fe(CN)_6$ were harvested by centrifugation and resuspended in PBS or fresh THY medium in the presence of $Fe(CN)_6$ (Table 2). Binding activity was only generated following incubation with the oxidant in conditioned medium (or heat-treated conditioned medium), which consisted of the cell-free supernatant fluids recovered following centrifugation of the overnight culture (Table 2). These results suggested that a secreted product of streptococcal metabolism plays a crucial role in the generation of binding activity. Streptococci secrete lactic acid as an end product of fermentation, and under the conditions used in this study, the pH of the medium will decrease from pH 7.5 for uninoculated media to pH 5.8 following entry into stationary phase. The addition of lactic acid to uninoculated medium to produce pH 5.8 was sufficient to restore the generation of binding activity following incubation with $Fe(CN)_6$ (Table 2). Lactic acid itself was not essential, since pH 5.8 generated by using HCl was also permissive for the generation of binding activity, and raising the pH of conditioned medium to pH 7.8 could block generation of binding activity (Table 2).

Titration revealed a strict dependence on pH for the modification reaction, with 50% of maximal binding activity obtained at pH 6.5 and no activity obtained above pH 7.0 (Fig. 5). Fibronectin binding was not affected if the actual binding assay was conducted at pH 5.8 (not shown); however, the binding assays were routinely conducted at pH 7.2 following exposure to conditions that promoted the binding activity (see Materials and Methods). Thus, the dependence on pH does not reflect a requirement for the actual binding of fibronectin to the component but rather is most likely required for the modification of the binding structure into its binding-competent form.

However, pH by itself was not sufficient, since incubation with the oxidant in PBS at pH 5.8 did not generate binding activity (Table 2). Preliminary fractionation experiments revealed that a low-molecular-weight compound was also likely required, since binding activity could be generated in the presence of a <3,000-molecular-weight fraction prepared from conditioned medium (Table 2). The observation that activation could be blocked in the presence of EDTA suggested that this substance was a divalent cation. The presence of the Fe chelator desferoxamine did not block the reaction, and the addition of either Mg^{2+}, Ca^{2+}, or Mn^{2+} to pH 5.8 PBS did not promote the generation of binding activity (Table 2). However, when Zn^{2+} (10 μM) was added to SAM1 cells in a pH 5.8 buffer with $Fe(CN)_6$, binding activity equivalent to that obtained in the presence of conditioned medium was observed (Table 2). Binding in the presence of Zn^{2+} could be blocked by the inclusion of EDTA, and the binding of fibronectin to Zn^{2+}-treated cells could be inhibited by the *E. coli* lysate (Table 2). Addition of the Zn^{2+}-specific chelator phenanthroline to conditioned medium could block activation (Table 2), providing additional evidence for a requirement for Zn^{2+}. Also, the addition of EDTA after exposure to permissive modifying conditions was as effective as adding EDTA prior to exposure, suggesting that Zn^{2+} is required for the binding of fibronectin to the modified component rather than for the modification reaction itself. Equivalent results were obtained when the streptococci were cultured in a chemically defined medium (44) instead of THY medium (not shown). Because of its dependence on Zn^{2+}, an oxidant, and pH, this non-protein F binding activity will be referred to as the ZOP pathway for fibronectin binding.

TABLE 2. Evaluation of reaction conditions[a]

Reaction medium	$Fe(CN)_6$	pH or addition(s)	Fibronectin binding[b]
Uninoculated THYB	−		−
	+		−
Conditioned THYB	−		−
	+		+++
Conditioned THYB, heat-treated	+		+++
PBS	+		−
Uninoculated THYB	+	pH 5.8, lactic acid	+++
	+	pH 5.8, HCl	+++
Conditioned THYB	+	pH 7.5	−
PBS	+	pH 5.8	−
Conditioned THYB,	+		++++
<3,000	−		−
Uninoculated THYB,	+	pH 7.5	−
<3,000	+	pH 5.8	++++
Conditioned THYB	+	1.0 mM EDTA	−
Conditioned THYB, <3,000	+	1.0 mM EDTA	−
Conditioned THYB	+	1.0 mM desferoxamine	+++
PBS	+	1.0 mM $MgSO_4$, pH 5.8	−
	+	1.0 mM $CaCl_2$, pH 5.8	−
	+	1.0 mM $MnCl_2$, pH 5.8	−
	+	10 μM $ZnSO_4$, pH 5.8	++++
	−	10 μM $ZnSO_4$, pH 5.8	−
	+	10 μM $ZnSO_4$, pH 5.8, 1.0 mM EDTA	−
	+	10 μM $ZnSO_4$, pH 5.8, inhibitor[c]	−
Conditioned THYB	+	1 mM phenanthroline	−

[a] SAM1 was cultured 18 h in liquid THY medium in the absence of 1 mM $Fe(CN)_6$ and resuspended in the indicated assay medium. Conditioned THYB consists of a supernatant of SAM1 cultured O/N in the indicated medium in the absence of $Fe(CN)_6$; <3,000 refers to an ultrafiltrate of conditioned THYB of <3,000 molecular weight. The reactions were incubated for 1 h at 37°C in the absence (−) or presence (+) of 1 mM $Fe(CN)_6$. Cells from the reaction were harvested by centrifugation and resuspended in PBS-T (pH 7.2), and their abilities to bind ^{125}I-fibronectin were determined.

[b] +++, binding equivalent (±5%) to the binding of SAM1 cultured O/N in THYB plus 1 mM $Fe(CN)_6$; ++++, 125 to 150% of control binding; −, binding no greater (±5%) than background values.

[c] Sonic lysate of *E. coli*, tested as an inhibitor of binding to treated cells, is described in the legend to Fig. 1.

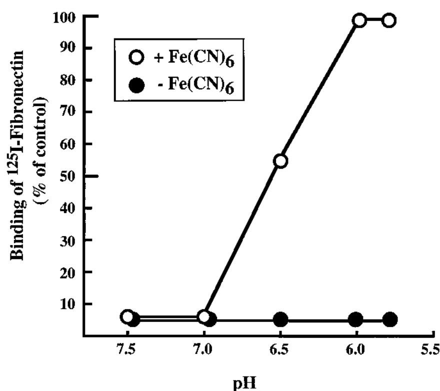

FIG. 5. Generation of binding activity is sensitive to pH. SAM1 cells from an overnight culture in liquid THY medium were resuspended in conditioned medium adjusted to the pHs indicated in the figure. Following a 1-h incubation at 37°C in the presence or absence of 1 mM $Fe(CN)_6$ as indicated, the abilities of the treated cells to bind fibronectin were determined. Binding is quantitated relative to the binding of SAM1 cultured overnight anaerobically in the presence of 1 mM $Fe(CN)_6$. The data reported represent mean values from a minimum of two independent experiments, which differed by less than 5%.

ZOP binding is protease resistant. Previous studies have suggested that streptococcal fibronectin binding correlates with strong surface hydrophobicity of the bacterial cells (1). However, JRS4, SAM1, and HSC5 cells cultured under aerobic conditions or in the presence of $Fe(CN)_6$ to promote activation of the ZOP binding pathway were not more hydrophobic than when cultured under oxygen-limiting conditions (not shown). In preliminary attempts to purify the ZOP binding activity, it was found that brief sonication could reduce the ability of treated cells to bind fibronectin by up to 90% compared with untreated cells. However, all attempts to isolate a fibronectin-binding protein from material released by sonication or from cell wall preparations or by several different extraction methods from whole cells or cell walls (including urea, phenol, LiCl, HCl, freeze-thaw, lysozyme, and mutanolysin) proved unsuccessful, suggesting that the ZOP fibronectin-binding structure is not a protein. This was further supported by testing the abilities of protease-treated cells to bind fibronectin. Trypsin treatment did not inhibit binding via the ZOP activity and actually substantially enhanced the ability of ZOP-expressing SAM1 cells to bind fibronectin (Fig. 6). In contrast, similar trypsin treatment inhibited the fibronectin-binding activity of protein F-expressing streptococci by greater than 90% (Fig. 6). Virtually identical results were obtained with chymotrypsin, pronase, and proteinase K (not shown).

Adherence to basement membrane. The ability of laminin to inhibit the binding of fibronectin by the ZOP pathway (see above) suggested that ZOP binding may promote the adherence of *S. pyogenes* to structures in the host that contains this extracellular matrix protein even though they do not contain large amounts of fibronectin. An example of this type of structure is basement membrane. The EHS sarcoma basement membrane (Matrigel) contains as its principal component laminin (24), and *S. pyogenes* SAM1 and SAM2 bound very poorly to this basement membrane when not expressing the ZOP binding activity (Fig. 7A and Table 3). However, the ability of both strains to adhere to basement membrane is dramatically enhanced following culture with $Fe(CN)_6$ to promote activation of the ZOP pathway (Fig. 7B and Table 3). Binding to basement membrane was inhibited by treatments which also inhibit fibronectin binding via the ZOP pathway (EDTA [Table 3]) and was not affected by treatments which were noninhibitory for fibronectin binding (BSA and Triton X-100 [Table 3]).

DISCUSSION

In this study, we have shown that the fibronectin-binding activity induced by aerobic growth of *S. pyogenes* is bimodal and consists of a binding component that we have designated ZOP in addition to the previously characterized protein F. However, unlike with protein F (45), O_2 does not induce the transcription of a fibronectin receptor. Rather, O_2 appears to be required for the modification of a heat- and protease-resistant surface component into a binding-competent form. The modification reaction is sensitive to pH, and the binding of fibronectin to the modified component requires Zn^{2+}. Unlike protein F, the ZOP binding activity has the ability to interact with extracellular matrix proteins in addition to fibronectin and can promote the interaction of *S. pyogenes* with basement membrane. Also unlike protein F, the binding of fibronectin to the ZOP binding activity can be inhibited by LTA.

LTA had been the first molecule implicated as the streptococcal receptor for fibronectin, principally on the basis of the ability of purified LTA to inhibit the binding of soluble fibronectin to intact streptococcal cells (1, 17, 37). However, the identification and cloning of several streptococcal fibronectin-binding proteins, including protein F (14), have resulted in some confusion over the possible role of LTA in fibronectin binding. The observation that LTA inhibits fibronectin binding via the ZOP pathway suggests that this binding activity may represent the activity that had been attributed to LTA. ZOP

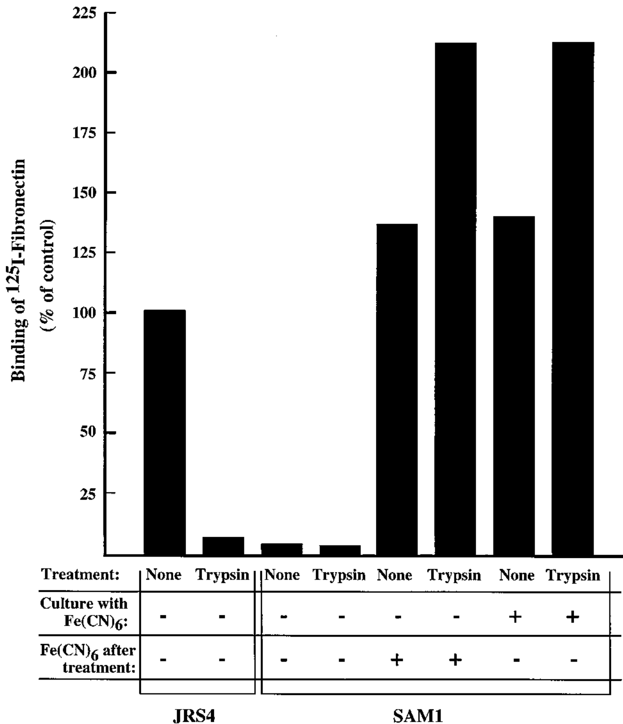

FIG. 6. ZOP binding activity resistant to treatment with proteases. JRS4 and SAM1 cultured in liquid THY medium in the presence (+) or absence (−) of 1 mM $Fe(CN)_6$ were treated with trypsin as indicated. Also as indicated, selected groups were incubated with $Fe(CN)_6$ following trypsin treatment. Binding is quantitated relative to the binding of JRS4 cultured anaerobically. The data reported represent mean values from a minimum of three independent experiments, which differed by less than 5%.

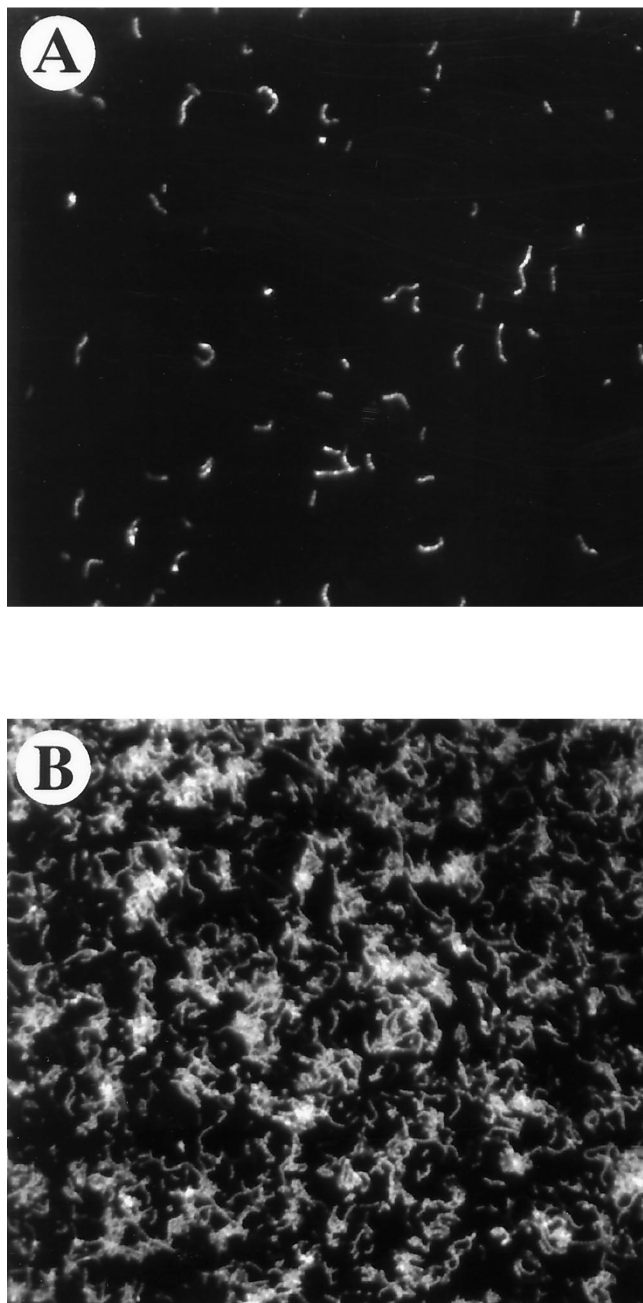

FIG. 7. ZOP binding activity promotes adherence to basement membrane. The ability of SAM1 cultured in the absence (A) or presence (B) of 1 mM $Fe(CN)_6$ to adhere to a basement membrane purified from the EHS sarcoma (Matrigel) is shown. Following washing to remove nonadherent bacteria, adherent streptococci were visualized by staining with acridine orange and fluorescent microscopy. Magnification, ×450.

binding does have several characteristics previously associated with the binding attributed to LTA, including stability to heat and proteases, a looser association with the cell surface, and inhibition by a broad spectrum of other extracellular matrix proteins (1, 5, 17, 37). However, it is not clear that LTA participates in ZOP binding, since in preliminary experiments, we found that LTA purified from streptococci grown under conditions non-permissive for ZOP activity was as effective in inhibiting binding as LTA purified from cells expressing ZOP activity, suggesting that LTA is not the molecule that becomes modified. Also, extracts of *E. coli* effectively inhibit binding even though *E. coli* does not produce LTA and the *E. coli* strain used does not bind fibronectin. Thus, while these inhibition studies provide insight into the mechanism by which the ZOP activity interacts with fibronectin, the inhibitory molecules may not reflect the actual receptor utilized by the intact streptococcal cells. Identification of the specific *E. coli* molecule responsible for inhibition and its comparison with the structure of LTA will likely yield useful clues as to the mechanism of ZOP-mediated binding.

TABLE 3. Adherence to basement membrane[a]

Strain	$Fe(CN)_6$	Adherence to basement membrane[b]			
		No addition	BSA	EDTA	Triton X-100
SAM1	+	+++	+++	−	+++
	−	−	−	−	−
SAM2	+	+++	+++	−	+++
	−	−	−	−	−

[a] The indicated strains were cultured in the presence (+) or absence (−) of 1 mM $Fe(CN)_6$. Adherence to purified EHS sarcoma basement membrane (Matrigel) was analyzed as described in Materials and Methods. Bacteria were visualized by staining with acridine orange and quantitated by microscopic examination. Bacteria were incubated with no addition, BSA (10 μg/ml), EDTA (10 mM), or Triton X-100 (0.1%) for 30 min prior to incubation with Matrigel.

[b] +++, >1,000 streptococcal chains per microscopic field; ++, 500 to 999 chains; +, 101 to 499 chains; −, <100 chains.

In examining the induction of fibronectin binding in response to O_2, it had previously been shown that both $Fe(CN)_6$ and the superoxide-generating agent methyl viologen could stimulate expression of fibronectin-binding activity of streptococcal cells grown under O_2-limited conditions (45). The data presented here indicate that the oxidizing agent $Fe(CN)_6$ exclusively stimulates the ZOP binding activity and does not induce expression of protein F. Preliminary transcriptional studies have confirmed that $Fe(CN)_6$ does not induce transcription of *prtF*, while methyl viologen is an effective inducer of *prtF* transcription and does not stimulate ZOP binding (13). Thus, two different aspects of exposure to an aerobic environment (external redox conditions and internal O_2^-) are involved in controlling the expression of two distinct adherence pathways which can recognize the same receptor (fibronectin). This capacity to specifically stimulate each pathway may also have contributed to some confusion concerning the mechanism(s) of streptococcal fibronectin binding.

While an aerobic environment modulates fibronectin binding by both protein F and ZOP, the mechanisms of control are quite different, involving transcriptional control of the expression of *prtF* versus posttranslational external modification of the ZOP binding component. As for the ZOP component described here, the interaction of human lipoproteins with receptors can also be profoundly influenced by oxidative modification, in that lipoproteins damaged by oxidation can bind to receptors on cells that do not have affinity for the nonoxidized forms of the lipoproteins (18, 39). It is of interest that only aged and presumably damaged serum lipoproteins, not freshly purified lipoproteins, were found to compete for LTA-mediated fibronectin binding to *S. pyogenes* (38). Also, LTA has recently been shown to bind to the same macrophage receptor that can recognize oxidized lipoproteins (10).

S. pyogenes may encounter several environments permissive for expression of ZOP binding activity during the course of infection. In the cutaneous tissues, *S. pyogenes* can initially transiently colonize the surface of the skin, an aerobic envi-

ronment of suitably low pH and of sufficient Zn^{2+} concentration (19) to be permissive for the generation of the ZOP binding activity. Disease is often initiated by the implantation of the bacterium into the deeper tissue by some type of mild trauma that breaches the integrity of the skin surface (46). The ZOP binding activity may then promote binding to fibronectin or other extracellular matrix proteins which may be exposed in the wound. Following entry into the tissue, streptococcal infections are characterized by intense inflammation and the accumulation of neutrophils. This environment may also be permissive for expression of the ZOP binding activity, since the pH of an inflammatory lesion can be reduced to levels which can support the ZOP modification reaction and since neutrophils can promote the release of Zn^{2+} from metalloproteins (11). Neutrophils also secrete H_2O_2 and myeloperoxidase, which together can generate a number of strong oxidants capable of oxidizing various cellular components, including lipoproteins (8). With its ability to promote the interaction of *S. pyogenes* with basement membrane, activation of the ZOP pathway at this stage of infection may aid the bacterium in invading deeper tissue.

ACKNOWLEDGMENTS

We are grateful for the technical assistance of Debra Weishaar. We thank William Goldman and Kathy Erwin for their gift of purified peptidoglycan and Ivo van de Rijn for his gift of CDM. The advice of Dan Goldberg and Bob Mecham is also sincerely appreciated.

This investigation was supported by the Monsanto/Searle research collaboration and Public Health Service grant AI38273-01 from the National Institutes of Health. M.C. is an Established Investigator of the American Heart Association.

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Editor: P. E. Orndorff