

## Resistance of a Tn4351-Generated Polysaccharide Mutant of *Porphyromonas gingivalis* to Polymorphonuclear Leukocyte Killing

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**In this study, we describe the development of an efficient transpositional mutagenesis system for *Porphyromonas gingivalis* using the *Bacteroides fragilis* transposon Tn4351. Using this system, we have isolated and characterized a Tn4351-generated mutant of *P. gingivalis* A7436, designated MSM-1, which exhibits enhanced resistance to polymorphonuclear leukocyte (PMN) phagocytosis and killing. *P. gingivalis* MSM-1 was initially selected based on its colony morphology; MSM-1 appeared as a mucoid, beige-pigmented colony. Analysis of *P. gingivalis* MSM-1 by electron microscopy and staining with ruthenium red revealed the presence of a thick ruthenium red-staining layer that was twice the thickness of this layer observed in the parent strain. *P. gingivalis* MSM-1 was found to be more hydrophilic than strain A7436 by hydrocarbon partitioning. Analysis of phenol-water extracts prepared from *P. gingivalis* A7436 and MSM-1 by Western (immunoblot) analysis and immunodiffusion with hyperimmune sera raised against A7436 and MSM-1 revealed the loss of a high-molecular-weight anionic polysaccharide component in extracts prepared from MSM-1. *P. gingivalis* MSM-1 was also found to be more resistant to PMN phagocytosis and intracellular killing than the parent strain, as assessed in a fluorochrome phagocytosis microassay. These differences were statistically significant ( $P < 0.05$ ) when comparing PMN phagocytosis in nonimmune serum and intracellular killing in nonimmune and immune sera. *P. gingivalis* MSM-1 was also more resistant to killing by crude granule extracts from PMNs than was *P. gingivalis* A7436. These results indicate that the increased evasion of PMN phagocytosis and killing exhibited by *P. gingivalis* MSM-1 may result from alterations in polysaccharide-containing antigens.**

*Porphyromonas gingivalis* (formerly *Bacteroides gingivalis*) has been implicated as a major pathogen in adult periodontitis (29). The host response to infection with *P. gingivalis* is characterized by elevated serum and local antibody response, with the cellular infiltrate at the site of destruction dominated by plasma cells and polymorphonuclear leukocytes (PMNs) (2). Despite evidence for a significant humoral and cellular response by the host, *P. gingivalis* appears to successfully colonize the periodontal pocket. Although a host protective role for antibody has not been demonstrated, the PMN is apparently host protective. This is evident when PMN function is defective, leading to rapid periodontal breakdown (58). The ability of *P. gingivalis* to evade phagocytosis in vitro has been well documented (6, 7, 52, 53) and may relate to the pathogenic potential of this organism in vivo (12).

The ability of pathogenic microorganisms to evade PMN phagocytosis and killing in many cases appears to result from alterations in the outer membrane as well as in the O-antigen-containing polysaccharide capsule (59). *P. gingivalis* produces a number of potential virulence factors that are believed to be crucial to the ability of this organism to colonize, invade the periodontal pocket, evade the host response, and cause disease (26). These include fimbriae, hemagglutinin, polysaccharide-containing capsule, lipopolysaccharide (LPS), outer membrane vesicles, and enzymatic activities that can perturb host defense mechanisms as well as initiate tissue destruction. The produc-

tion of one or several of these virulence factors is believed to play a crucial role in the ability of *P. gingivalis* to evade the host response. We have shown previously that the production of C3 and immunoglobulin G (IgG) proteases may be associated with resistance of *P. gingivalis* to PMN-mediated phagocytosis (5). The cell surface of *P. gingivalis* has also been postulated to play a primary role in the interactions of this organism with PMNs (36); increased encapsulation of different *P. gingivalis* strains has been associated with a reduction in phagocytosis (53). Although these in vitro studies have correlated the presence of the *P. gingivalis* polysaccharide capsule with resistance to PMN-mediated phagocytosis, the exact role of the *P. gingivalis* polysaccharide-containing capsule remains speculative.

The use of genetically defined strains of *P. gingivalis* is essential for understanding the specific mechanisms employed for resistances to PMN phagocytosis. Although this organism produces a number of potential virulence factors, an analysis of these factors at the genetic level has been hampered by a lack of available genetic tools (15). The majority of genetic studies in *Bacteroides* spp. to date have involved the colonic species (3, 16, 31, 39, 41), partly because human colonic *Bacteroides* spp. are among the most oxygen tolerant of all obligate anaerobes. Shoemaker et al. (48) have reported on an efficient transpositional mutagenesis system for the intestinal *Bacteroides* spp. utilizing Tn4351 (3, 16). Recently, several reports have described the introduction of the *Bacteroides fragilis* transposon Tn4351 into *P. gingivalis* at frequencies ranging between  $10^{-7}$  and  $10^{-10}$  (8, 18, 35). Although these initial reports have confirmed the introduction of Tn4351 into *P. gingivalis*, higher

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frequencies for transposition of Tn4351 must be obtained for Tn4351 to be used as an efficient mutagenesis tool for *P. gingivalis*. In this study, we describe conditions which yield maximal transfer of Tn4351 into *P. gingivalis* and confirm the random insertion of Tn4351 into the *P. gingivalis* chromosome. Transpositional mutagenesis was subsequently used to isolate a mutant of *P. gingivalis* A7436, designated MSM-1, which exhibits an increased resistance to phagocytosis and killing by human PMNs. Analysis of *P. gingivalis* MSM-1 by electron microscopy and initial characterization of purified polysaccharide indicate an apparent loss of an anionic polysaccharide antigen, with an associated overproduction of O-side-chain polysaccharide.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *P. gingivalis* A7436 and 381 (12), *Escherichia coli* HB101(R751:Tn4351), and *Bacteroides thetaiotaomicron* 5482 (48) were used in these studies. *P. gingivalis* and *B. thetaiotaomicron* cultures were typically maintained on anaerobic blood agar (ABA; Remel, Lenexa, Kans.) at 37°C in an anaerobe chamber (Coy Laboratory Products Inc.) with 85% N<sub>2</sub>, 5% H<sub>2</sub>, and 10% CO<sub>2</sub>. After incubation at 37°C for 3 days (*P. gingivalis*) or 1 day (*B. thetaiotaomicron*), cultures were inoculated into Anaerobic Broth MIC (Difco Laboratories, Detroit, Mich.), Schaedler broth (SB; Difco Laboratories), or basal medium supplemented with hemin (1.0 µg/ml) (BM; Trypticase peptone, 10 g; tryptophan, 0.2 g; NaCl, 2.5 g; sodium sulfite, 0.1 g; and cysteine, 0.4 g [per liter]) and incubated at 37°C under anaerobic conditions for 24 h. Growth was monitored as the A<sub>660</sub> (Beckman DU8 spectrophotometer). *P. gingivalis* MSM-1 was typically maintained on erythromycin (Em, 1.0 µg/ml)-containing medium; *E. coli* was maintained on Luria broth (LB) plates containing tetracycline (Tc) (10.0 µg/ml).

**Transpositional mutagenesis and isolation of *P. gingivalis* MSM-1.** Tn4351 was introduced into *P. gingivalis* A7436 or 381 by conjugation using a broad adaptation of the procedure of Shoemaker et al. (48), which was specifically modified for *P. gingivalis*. Introduction of Tn4351 into *B. thetaiotaomicron* was performed as described before (48). Tn4351 is carried on the broad-host-range plasmid R751, which is capable of transferring itself to a wide range of organisms. However, R751 is not capable of replication in *P. gingivalis*, and once the plasmid is transferred, Tn4351 excises and integrates into the host chromosome. Tn4351 carries resistance to Em and Tc; Em<sup>r</sup> functions in *Bacteroides* spp., while Tc<sup>r</sup> is expressed only in *E. coli*.

*P. gingivalis* was subcultured from an ABA plate into 10 ml of SB and grown anaerobically to an A<sub>660</sub> of between 0.3 and 0.5 (early logarithmic growth). *E. coli* was subcultured into LB and grown aerobically at 37°C to an A<sub>660</sub> of 0.2. Samples were removed (1.0 ml of *P. gingivalis* and 0.1 ml of *E. coli*), centrifuged at 12,000 × g, and resuspended in 0.05 ml of SB or LB, respectively. Conjugations were performed under anaerobic conditions in 125-ml flasks containing 25 ml of reduced blood agar on which a 0.45-µm HAWP filter (Millipore) was aseptically placed. *P. gingivalis* and *E. coli* cultures were spotted on filters, the flasks were tightly stoppered and removed from the chamber, and 1.0 ml of air was added to each flask with a hypodermic needle and syringe. Stoppered flasks were incubated overnight (18 to 24 h) at 37°C aerobically. *P. gingivalis* and *E. coli* cultures only were also spotted onto filters as described above and served as controls. Following incubation, filters were removed from flasks, cells were washed off by vortexing in 2.0 ml of SB, and cultures were incubated at room temperature (RT) for an additional 45 min. Cultures were serially diluted and plated on selective medium (ABA containing geneticin [Gn, 75 to 50.0 µg/ml] and Em [1.0 µg/ml]). *Bacteroides* spp. are naturally resistant to aminoglycosides, while *E. coli* is susceptible even when grown anaerobically (48). The CFU of input cultures was determined by plating *E. coli* on LB plates containing Tc and *P. gingivalis* on ABA plates containing Gn. Plates were incubated anaerobically for 7 to 10 days, and potential transconjugants were restreaked on selective medium and incubated anaerobically at 37°C; transconjugants were typically maintained on selective medium and grown anaerobically. *P. gingivalis* MSM-1 was initially selected for further characterization based on its mucoid/nonpigmented phenotype when grown on ABA and was typically maintained on selective medium.

To confirm the insertion of Tn4351 into the *P. gingivalis* chromosome, chromosomal DNA was prepared from *P. gingivalis* MSM-1 and eight randomly selected transconjugants (25), digested with *EcoRI*, *HindIII*, or *PstI* according to the manufacturer's instructions (Stratagene, Inc.) and electrophoresed on a 0.8% agarose gel in 1 × TBE buffer at 20 V for 16 h. Gels were probed by Southern blot analysis (50) with pVOH1 or R751 (48). pVOH1 and pR751 were isolated from *E. coli* by a modification of the alkaline lysis method (25).

**Electron microscopy.** Early-logarithmic-phase cultures of *P. gingivalis* were centrifuged (10,000 × g for 5 min) and washed twice in phosphate-buffered saline (PBS). Concentrated cultures were fixed at RT for 2 h with 3.6% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), followed by secondary fixation at RT in 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) for 1 h.

To maximize preservation of polysaccharide-containing material, ruthenium red (0.075%) and lysine (55 mM) were added to the glutaraldehyde fixative (4). Grids were viewed in a JEM-1200 EX electron microscope.

**Hydrophobicity assay.** The hydrophobicity of *P. gingivalis* MSM-1 was compared with that of *P. gingivalis* A7436 as previously described (37). Briefly, *P. gingivalis* MSM-1 and A7436 and *Actinobacter calcoaceticus* ATCC 31012 (a hydrophobic control organism) were suspended in PUM buffer (37), and the A<sub>660</sub> of all three organisms was adjusted to 0.5. The A<sub>660</sub> was again measured in the aqueous phase after the addition of increasing volumes of hexadecane. The degree of hydrophilicity was expressed as the percent change in A<sub>660</sub> as a function of added hexadecane (37).

**Polysaccharide-containing antigen preparation.** Polysaccharide-containing antigen preparations were prepared from *P. gingivalis* A7436 and MSM-1 by two procedures, a potassium hydroxide (KOH) preparation and a phenol-water (PW) preparation. Both procedures provide a mixture of polysaccharide and lipopolysaccharide (LPS); however, the KOH preparation appears to enrich for polysaccharide for some strains of *P. gingivalis*. Briefly, *P. gingivalis* bacterial cells were suspended at ~0.2 g (wet weight) per ml in 0.15 M NaCl–0.05 M KOH–0.01 M EDTA and stirred at RT for 2 h. Supernatants were obtained by centrifugation and dialyzed overnight against deionized water. Following dialysis, solutions were brought to 0.15 M NaCl–0.005 M Tris-HCl (pH 7.5)–4 mM Mg<sup>2+</sup>–1 mM Ca<sup>2+</sup> and treated with DNase I (0.01 mg/ml) and RNase A (0.04 mg/ml) for 2 h at 37°C and then with proteinase K (0.04 mg/ml) for 1 h at 20°C. Samples were then dialyzed and lyophilized. For the PW preparation, bacterial cells were suspended at ~0.2 g (wet weight) per ml in water and extracted with an equal volume of 90% phenol with stirring for 20 min at 65 to 70°C. The aqueous phase was removed by centrifugation and dialyzed against water for 2 days. Samples were then treated with DNase I, RNase A, and proteinase K as for the KOH preparation.

**SDS-PAGE and Western blot analysis.** For whole-cell preparations, *P. gingivalis* A7436 and MSM-1 were grown on ABA, and cells were removed, washed, and resuspended in 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4) with 50 µM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by boiling in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer for 10 min. KOH and PW extracts and whole-cell lysates were separated by SDS-PAGE by the method of Laemmli (23) with a 10% resolving gel and a 3% stacking gel. Prestained electrophoresis molecular weight markers were used as standards. Gels were stained with either Coomassie blue (Sigma Chemical Co., St. Louis, Mo.), alcian blue (27), or a silver stain with periodate (56).

*P. gingivalis* A7436 and MSM-1 antigens were detected by Western blot analysis with hyperimmune rabbit sera raised to *P. gingivalis* A7436 and MSM-1 by hyperimmunization in rabbits as previously described (6). Following separation by SDS-PAGE, antigens were transferred to nitrocellulose by Western immunoblotting (55). Nitrocellulose filters were blocked with 1% bovine serum albumin (BSA) and incubated with sera (1:1,000) at RT for 1 h. Following incubation with primary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma Chemical Co.) was added. Immunoblots were developed by the addition of TAB (3,3',5,5'-tetramethylbenzidine) membrane peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

**Immunoelectrophoresis.** PW extracts from A7436 and MSM-1 were dissolved at 2 mg/ml, and immunoelectrophoresis was performed in 0.8% agarose in barbital-acetate buffer, pH 8.2. Wells contained 8 µl of extract, and the time of electrophoresis was determined by migration of the bromophenol blue tracking dye to the anode (2 to 3 h) at 120 V. Following electrophoresis, troughs were filled with immune rabbit sera prepared against formalin-fixed whole bacterial cells (A7436 and MSM-1) and allowed to develop for 24 h at 4°C in a humidified container.

**Fluorescent phagocytosis and killing microassay.** PMN phagocytosis and killing of *P. gingivalis* A7436 and MSM-1 were monitored as previously described by Cutler et al. (6). Briefly, bacteria labeled with 1.0 mg of the fluorochrome dye 4',6-diamidino-2-phenylindole (DAPI) per ml in distilled H<sub>2</sub>O were opsonized for 15 min at 37°C with either 20% nonimmune rabbit serum or 20% hyperimmune serum. Human PMNs were added at a bacterium-PMN ratio of 10:1, followed by the addition of the viability dye propidium iodide (PI) (5.0 µg/ml). After incubation for 15 min at 37°C with agitation, 40-µl aliquots were removed, 4 µM acridine orange (AO) was added (to observe the intracellular architecture of PMNs), and samples were processed by cytofluorimetry (Shandon; Lipshaw Inc., Pittsburgh, Pa.). Duplicate slides were prepared for each experimental condition, and all slides were visualized on a Zeiss fluorescence microscope. Slides were coded and analyzed in a double-blinded design, so that the examiner was unaware of the identity of the slide. Parameters monitored included PMN phagocytosis (mean percentage of PMNs actively phagocytosing *P. gingivalis*), number of *P. gingivalis* per PMN (mean number of *P. gingivalis* internalized per phagocytic PMN), and killing (mean percent PI-positive *P. gingivalis* per phagocytic PMN). Adherence was differentiated from phagocytosis as previously described (6).

**PMN granule killing assay.** Human PMNs were obtained from healthy volunteers (6), and granule extracts were prepared as described before (28) in 0.5% cetyltrimethylammonium bromide (CTAB)–0.02 M sodium acetate–0.2 M NaCl, pH 4.7. The suspension was dialyzed in 0.02 M sodium acetate–0.2 M NaCl (pH 4.7) buffer for 48 h to remove the CTAB, followed by concentration in an

TABLE 1. Conjugal transfer of Tn4351<sup>a</sup>

Recipient strain	Input of recipient (CFU)	Donor/recipient ratio	Trans-conjugants (CFU)	Conjugation frequency <sup>b</sup>
<i>P. gingivalis</i>				
A7436	$6.3 \times 10^5$	1:500	$3.4 \times 10^1$	$5.4 \times 10^{-5}$
A7436	$4.5 \times 10^8$	1:30	$6.0 \times 10^3$	$1.3 \times 10^{-5}$
A7436	$1.5 \times 10^8$	1:1.5	$1.3 \times 10^3$	$8.6 \times 10^{-6}$
381	$6.0 \times 10^8$	1:2	$1.13 \times 10^3$	$1.9 \times 10^{-6}$
<i>B. thetaiotaomicron</i>				
5482 <sup>c</sup>	$1.9 \times 10^5$	1:5	$2.5 \times 10^1$	$1.3 \times 10^{-4}$

<sup>a</sup> *E. coli* HB101(R751:Tn4351) was used as the donor in conjugal matings at an input of  $10^7$  to  $10^8$  CFU. Results are from one experiment and are representative of at least two separate experiments.

<sup>b</sup> Conjugation frequency is expressed as CFU of transconjugants per CFU of input recipients per milliliter.

<sup>c</sup> Conjugal matings were performed as previously described (16).

Amicon Centriprep (final concentration, 100  $\mu$ g/ml). Early-logarithmic-phase cultures of *P. gingivalis* A7436 or MSM-1 (0.5 ml) were incubated with 0.1 to 0.2 ml of granule extract preparations and 0.4 ml of 0.01 M sodium phosphate, pH 7.0, containing 0.01 M NaCl. Control cultures contained 0.5 ml of buffer only. All cultures were incubated for 3 h at 37°C under anaerobic conditions. Viable bacterial cell counts were determined at 0 and 3 h by serial dilution and plating and expressed as CFU per milliliter.

## RESULTS

**Transposition of Tn4351 in *P. gingivalis*.** In initial studies, we sought to optimize conditions for conjugal transfer of Tn4351 into *P. gingivalis* and to maximize growth of *P. gingivalis*. We found that early-logarithmic-phase cultures as well as a minimal amount of air (i.e., at least 1.0 ml per flask) during conjugation, were essential (data not shown). In addition, we also determined that *P. gingivalis* strains were differentially sensitive to Em and Gn (data not shown). The use of Millipore filters was also found to significantly increase the conjugation frequency (data not shown). As shown in Table 1, the overall frequency of transposition of Tn4351 in *P. gingivalis* observed in this study was  $1.3 \times 10^{-5}$  to  $1.9 \times 10^{-6}$ . Although Shoemaker et al. (48) typically use a donor-to-recipient ratio of 1:5 for *B. thetaiotaomicron*, we were able to achieve higher conjugation frequencies for *P. gingivalis* by increasing the donor-recipient ratio (Table 1). The highest frequencies were obtained at donor-recipient ratios of 1:30 and 1:500. The increased frequencies obtained with these ratios may result from the increase in viable *P. gingivalis* capable of serving as recipients for Tn4351.

The frequency of transposition of Tn4351 in *P. gingivalis* 381 was lower than that obtained for strain A7436 at similar donor-recipient ratios, perhaps due in part to different restriction systems. Although the transposition frequencies of Tn4351 in *P. gingivalis* A7436 were slightly lower than previously reported for *Bacteroides uniformis* and *B. thetaiotaomicron* (16, 39, 40, 48), by modification of the original protocol (48), we were able to increase the transposition frequency  $10^1$ - to  $10^5$ -fold above that previously reported for *P. gingivalis* (8, 18, 35). The increase in transfer frequency may reflect the optimal mating conditions used (i.e., the increased number of recipients, the use of filters for mating, and the introduction of air during the conjugal mating). It has been reported that aerobic filter matings between *E. coli* and the colonic *Bacteroides* spp. results in a 50-fold increase in transfer frequency compared with that in matings performed anaerobically (48).

Transfer of *P. gingivalis* A7436 Em<sup>r</sup> transconjugants (including MSM-1; see below) to nonselective medium for 10 sequential passages did not result in the loss of Em<sup>r</sup>, indicating that

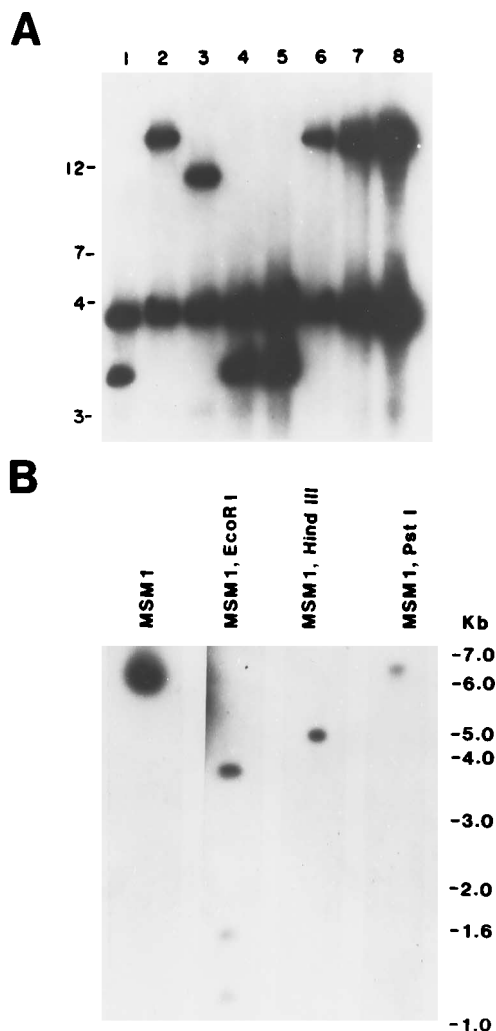


FIG. 1. Southern blot hybridization analysis of *P. gingivalis* transconjugants. (A) Chromosomal DNA from eight independent Em<sup>r</sup> Gn<sup>r</sup> transconjugants of *P. gingivalis* A7436 (lanes 1 to 8) were digested with *Eco*RI, separated by agarose gel electrophoresis, and transferred by Southern blot to Hybond N<sup>+</sup> nylon membranes. Molecular sizes (in kilobases) are indicated on the left. (B) *P. gingivalis* MSM-1 genomic DNA was digested with *Eco*RI, *Hind*III, or *Pst*I, separated by agarose gel electrophoresis, and transferred by Southern blot as above. Lane MSM-1, undigested DNA. Size markers (in kilobases) are indicated on the right. All membranes were hybridized with [<sup>32</sup>P]pVOH1 labeled by nick translation (25). We did not observe hybridization of *P. gingivalis* A7436 DNA with pVOH1 or R751 as the probe (data not shown).

Tn4351 was stably maintained in *P. gingivalis* in vitro (data not shown). In addition, we found that in two different Tn4351-generated *P. gingivalis* A7436 mutants (MSM-1 [see below] and MSM-3 [unpublished data]), Tn4351 was stable under nonselective pressure in vivo; using the mouse subcutaneous chamber model (12), we found that *P. gingivalis* MSM-1 and MSM-3 stably maintained Em<sup>r</sup> for up to 30 days postinoculation.

The presence of Tn4351 in transconjugants was initially confirmed by colony hybridization with pVOH1 (data not shown). To confirm the insertion of Tn4351 into the *P. gingivalis* chromosome, eight randomly selected transconjugants were analyzed by Southern blot analysis with pVOH1. To examine the possible insertion of R751 sequences in *P. gingivalis* transconjugants, Southern blots were also probed with R751. As shown in Fig. 1A, only one copy of Tn4351 was found in the chromo-

some of the *P. gingivalis* A7436 transconjugants. A simple insertion of Tn4351 is indicated by the strongly hybridizing 3.8-kb *Eco*RI fragment plus the additional hybridizing *Eco*RI fragment representing one junction fragment. *Eco*RI digestion also leaves less than 10 bases of IS4351 (48), which was not visible on this Southern blot. In three of the eight transconjugants examined, the junction fragments were of different sizes, indicating that the sites of insertion were different (Fig. 1). We did not detect hybridization of R751 to chromosomal DNA from the eight *P. gingivalis* transconjugants (data not shown).

**Isolation and initial characterization of MSM-1.** The Tn4351 insertional mutant *P. gingivalis* MSM-1 was initially selected for further characterization based on its mucoid/nonpigmented phenotype when grown on ABA. The presence of a single copy of Tn4351 in MSM-1 was confirmed by Southern analysis (Fig. 1B). *P. gingivalis* MSM-1 DNA harbored single *Hind*III and *Pst*I restriction fragments (representing the entire transposon) when pVOH1 was used as a probe. Southern blots hybridized with R751 failed to show hybridization of R751 to chromosomal DNA from *P. gingivalis* MSM-1 (data not shown), indicating that only Tn4351 had integrated into the chromosome.

From its nonpigmented colony phenotype, we reasoned that *P. gingivalis* MSM-1 may be defective in hemin utilization or storage. However, *P. gingivalis* MSM-1 exhibited the same growth kinetics as *P. gingivalis* A7436 when supplemented with hemin as an iron source (data not shown). *P. gingivalis* MSM-1 also achieved the same final cell yield as *P. gingivalis* A7436 (data not shown). Growth of MSM-1 in complex medium (SB or AB) was identical to that of strain A7436 with respect to generation time and final cell yield (data not shown).

**Analysis of *P. gingivalis* MSM-1 by electron microscopy.** Since we had initially selected *P. gingivalis* MSM-1 based on differences in colony morphology, we chose to next examine the cell surface of *P. gingivalis* MSM-1 by electron microscopy. From the mucoid phenotype, one possibility was that *P. gingivalis* MSM-1 could be altered in the production of a polysaccharide-containing capsule. The parent strain *P. gingivalis* A7436 produces a thick, electron-dense polysaccharide layer, as shown in Fig. 2A. The presence of this electron-dense polysaccharide layer was enhanced by using ruthenium red in the fixative. Analysis of MSM-1 by electron microscopy also revealed the presence of a thick ruthenium red-staining layer; however, this layer appeared to be denser than that of the parent strain (Fig. 2B). These results were consistent when four separate preparations of *P. gingivalis* A7436 and MSM-1 were viewed by electron microscopy (data not shown). Measurements of the thickness of this ruthenium red-staining layer in over 50 individual micrographs indicated that the average thickness of the *P. gingivalis* MSM-1 ruthenium red-staining layer was 50 nm; the average thickness of this layer in *P. gingivalis* A7436 was 26 nm. Therefore, our electron microscopic analysis data indicate that *P. gingivalis* MSM-1 produces a polysaccharide-containing layer that is approximately twice the thickness of this layer in strain A7436.

**Hydrophobicity analysis.** Haapasalo et al. (17) recently demonstrated that strains of *P. gingivalis* with a thick polysaccharide-containing capsule are more hydrophilic, while strains with a thin capsule are more hydrophobic. From the increased production of the polysaccharide-containing layer in *P. gingivalis* MSM-1 as seen by electron microscopy, we next examined the hydrophobicity of *P. gingivalis* MSM-1 in a hexadecane assay. Compared with the hydrophobic strain *A. calcoaceticus*, *P. gingivalis* A7436 and MSM-1 were found to be more hydrophilic (Fig. 3). In addition, we found that *P. gingivalis* MSM-1 was more hydrophilic than A7436. Although this difference was

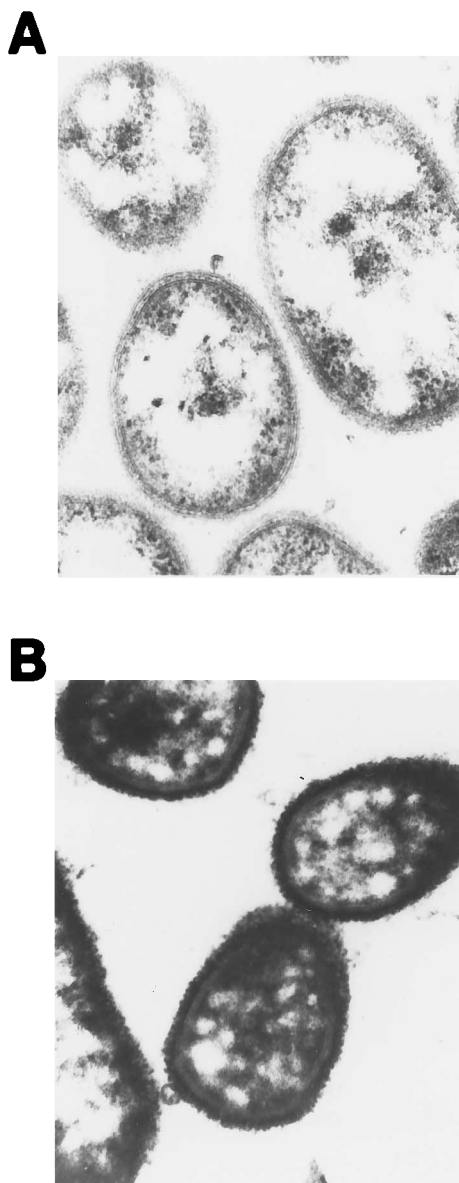


FIG. 2. Electron micrographs of *P. gingivalis* A7436 (A) and MSM-1 (B). Magnification,  $\times 180,000$ .

small, it was statistically significant when 10  $\mu$ l of hexadecane was added.

**Characterization of polysaccharide-containing antigen from *P. gingivalis* A7436 and MSM-1.** To examine possible antigenic differences in the polysaccharide-containing antigens produced by *P. gingivalis* A7436 and MSM-1, we examined the antigenic profile of purified polysaccharide-containing preparations using rabbit hyperimmune sera raised to *P. gingivalis* A7436 and MSM-1. The results of this analysis are depicted in Fig. 4. From the observed antigenic profile, the two different extraction procedures (KOH and PW) appear to provide a similar mix of antigens in both A7436 and MSM-1, as assessed by Western blot analysis (Fig. 4). However, we observed differences in the antigenic profile of extracts prepared from MSM-1 compared with extracts prepared from A7436. Of particular interest was the absence of a high-molecular-weight antigen

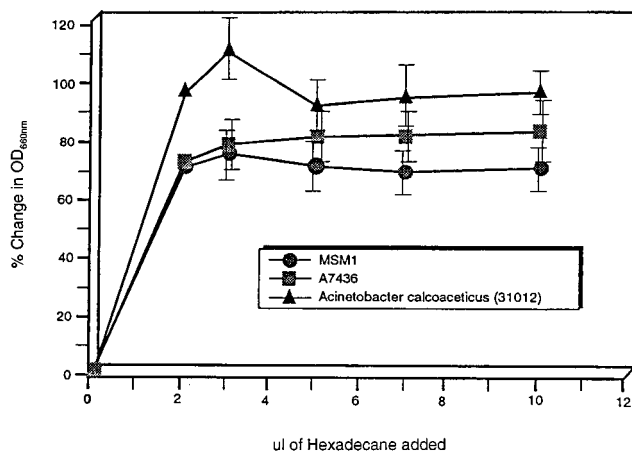


FIG. 3. Hydrophilicity profile for *P. gingivalis* A7436 and MSM-1. Assays were performed as described in Materials and Methods, and the degree of hydrophilicity is expressed as the percent change in optical density (OD) as a function of added hexadecane. OD units were converted to the percent change in OD according to the following formula:  $\{[(\text{original OD}) - (\text{new OD after hexadecane})] / (\text{original OD})\} \times 100$ . Results are presented as the mean  $\pm$  standard deviation for three separate experiments.

(>180 kDa) in PW and KOH extracts from MSM-1 (Fig. 4A and B). This antigen was not recognized in PW and KOH extracts from *P. gingivalis* A7436 when reacted with anti-MSM-1 serum (Fig. 4B). This antigen was also absent when polysaccharide-containing extracts prepared from *P. gingivalis* MSM-1 were stained with alcian blue, which specifically stains polysaccharide (data not shown). However, we did not detect differences when polysaccharide-containing preparations were stained with a silver stain which stains LPS (data not shown). In addition, both anti-A7436 and anti-MSM-1 sera reacted similarly when examined in Western blots of whole-cell lysates of A7436 and MSM-1 (data not shown).

**Immunoelectrophoresis.** Immunoelectrophoresis analysis was performed on the PW extracts prepared from *P. gingivalis* A7436 and MSM-1, and the results are shown in Fig. 5. The PW preparation from strain A7436 demonstrated the presence of a anionic component that was not present in the extract prepared from strain MSM-1; this was obvious when anti-A7436 serum was used. This anionic component most likely corresponds to the high-molecular-weight polysaccharide antigen detected in extracts of A7436 by Western blot analysis and alcian blue staining (Fig. 4). When MSM-1-specific anti-serum was used, we observed a faint precipitin band in PW extracts from A7436 (Fig. 5). In contrast, only one band was observed in PW extracts from MSM-1; this band may represent polysaccharide-containing LPS (45). Taken together, these results indicate that MSM-1 is missing a high-molecular-weight polysaccharide-containing anionic antigen.

**PMN phagocytosis and killing of *P. gingivalis* A7436 and MSM-1.** The observed difference in the polysaccharide-containing layer and the increased hydrophilicity of *P. gingivalis* MSM-1 prompted us to characterize the interactions between *P. gingivalis* MSM-1 and human PMNs. For these studies, we employed an in vitro fluorochrome microassay routinely used in our laboratory (6, 7). As shown in Fig. 6, we found that PMN phagocytosis for both strains A7436 and MSM-1 was extremely low, as was the number of *P. gingivalis* per PMN (Fig. 7). Interestingly, we also observed a difference when comparing MSM-1 and A7436; PMN phagocytosis for *P. gingivalis* MSM-1 was significantly lower than that observed for *P. gingivalis*

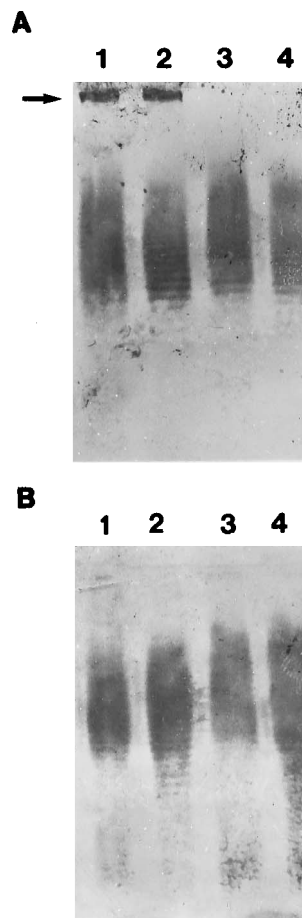


FIG. 4. Immunoblot analysis of KOH and PW extracts from *P. gingivalis* A7436 and MSM-1. Polysaccharide-containing antigen preparations from *P. gingivalis* A7436 and MSM-1 were examined by immunoblot analysis with rabbit antisera to strains A7436 and MSM-1. (A) Anti-*P. gingivalis* A7436; (B) anti-*P. gingivalis* MSM-1. Lanes: 1, A7436, KOH extract; 2, A7436, PW extract; 3, MSM-1, KOH extract; 4, MSM-1, PW extract. The arrow in panel A indicates the Ag present in A7436 KOH and PW extracts.

A7436 when nonimmune serum was used. These results indicate that *P. gingivalis* MSM-1 may interact less readily with human PMNs than *P. gingivalis* A7436. We also observed a difference in the number of *P. gingivalis* per PMN when

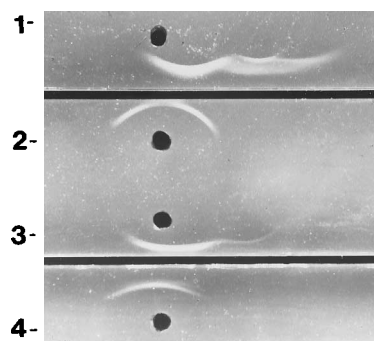


FIG. 5. Immunoelectrophoresis of PW extracts from *P. gingivalis* A7436 and MSM-1. PW extracts from *P. gingivalis* A7436 and MSM-1 were examined by immunoelectrophoresis with rabbit antisera to strains A7436 (top trough) and MSM-1 (bottom trough). 1 and 3, A7436 extract; 2 and 4, MSM-1 extract.

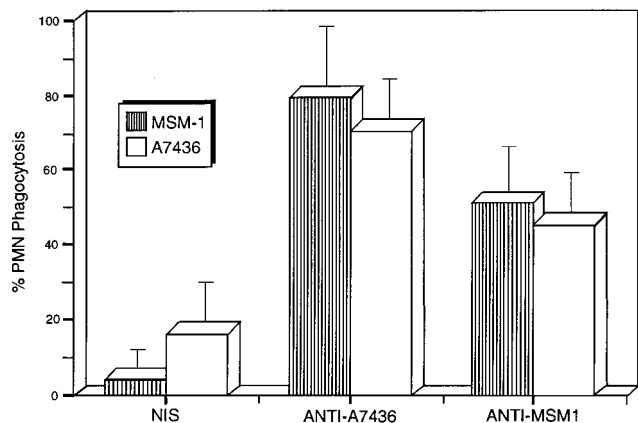


FIG. 6. PMN phagocytosis of *P. gingivalis* A7436 and MSM-1. The percentage of PMNs that were phagocytosing *P. gingivalis* was determined as described in Materials and Methods. Results are presented as the mean  $\pm$  standard deviation for three separate experiments;  $P < 0.05$ .

MSM-1 was incubated with nonimmune serum (Fig. 7). However, no differences in PMN phagocytosis or the number of *P. gingivalis* per PMN were observed when either anti-A7436 or anti-MSM-1 serum was used (Fig. 6 and 7). We also found that anti-A7436 serum was a much better opsonin for both *P. gingivalis* A7436 and MSM-1 (Fig. 6 and 7).

Although we did not detect significant differences in PMN phagocytosis and the number of *P. gingivalis* per phagocytic PMN with antibody-containing serum (anti-A7436 and anti-MSM-1), we did detect differences in PMN killing between *P. gingivalis* A7436 and MSM-1 (Fig. 8). Using nonimmune, anti-A7436, and anti-MSM-1 sera, we observed a statistically significant ( $P < 0.05$ ) difference in the number of dead bacteria within PMNs for *P. gingivalis* A7436 and MSM-1. This was most obvious when anti-MSM-1 serum was used as an opsonin (Fig. 8). PMN killing for *P. gingivalis* A7436 with anti-MSM-1 serum was 70%; in contrast, PMN killing for MSM-1 with anti-MSM-1 serum was 24%. Thus, *P. gingivalis* MSM-1 appears to be resistant to PMN killing, as assessed in this assay.

**Bactericidal assay.** The data presented in Fig. 8 indicated

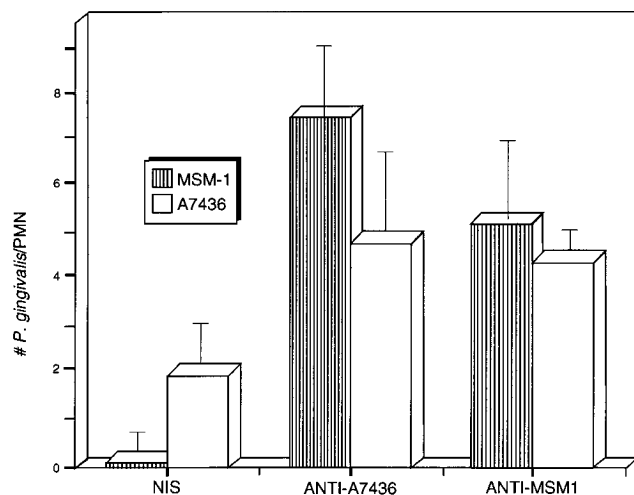


FIG. 7. Number of *P. gingivalis* A7436 and MSM-1 per PMN. The number of *P. gingivalis* phagocytosed per PMN was determined as described in Materials and Methods using the fluorochrome assay. Results are presented as the mean  $\pm$  standard deviation for three separate experiments;  $P < 0.05$ .

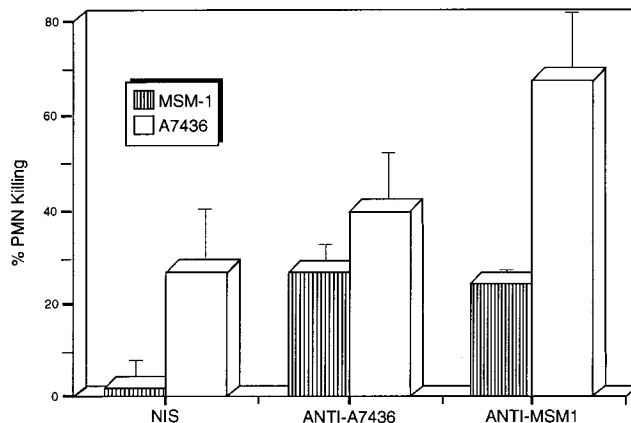


FIG. 8. Killing of *P. gingivalis* A7436 and MSM-1. Killing of *P. gingivalis* by nonimmune serum (NIS), anti-A7436 serum, and anti-MSM-1 serum was determined in the fluorochrome assay. PMN killing refers to the percentage of PMNs that are associated with dead *P. gingivalis* cells. Results are presented as the mean  $\pm$  standard deviation for three separate experiments;  $P < 0.05$ .

that *P. gingivalis* MSM-1 exhibited a greater resistance to killing once inside the PMN than did *P. gingivalis* A7436. Although previous growth and killing studies have confirmed that the permeability of *P. gingivalis* to PI is a valid indicator of cell death (6, 7, 8), we confirmed these results using an in vitro plating assay. For these experiments, we examined the bactericidal activity of crude lysosomal constituents obtained from human neutrophils against *P. gingivalis* A7436 and MSM-1. *P. gingivalis* A7436 and MSM-1 were incubated with crude granule fractions, and the bactericidal activity was determined following plating of viable bacteria. Incubation of *P. gingivalis* A7436 with PMN granule extract preparations (10 or 20  $\mu\text{g/ml}$ ) resulted in a 100-fold reduction in viability (Table 2). In contrast, *P. gingivalis* MSM-1 was not killed by granule extracts under similar conditions. These results confirmed our results obtained in the PMN fluorochrome microassay and support the increased resistance of *P. gingivalis* MSM-1 to killing by PMN antimicrobial enzymes.

## DISCUSSION

The use of genetic approaches for the study of *P. gingivalis* has been hampered by a lack of genetic exchange systems suitable for the manipulation of these organisms. In this study, we have developed an efficient mutagenesis system for *P. gingivalis* using the transposon Tn4351. Although previous reports have indicated that multiple insertions of Tn4351 may occur in *P. gingivalis* (8, 18), this was not observed in the studies reported here using *P. gingivalis* A7436. In addition, we did not detect the integration of the conjugal plasmid R751 into *P. gingivalis* A7436. The high frequency of transposition, together with the observed stability of the insertions, indicates that Tn4351 mutagenesis is a valuable tool for examining a variety of mutations in *P. gingivalis*. As shown here, the isolation and

TABLE 2. Killing of *P. gingivalis* with human PMN granule extract

Strain	Viability (CFU/ml)			
	Control	Treated (10 $\mu\text{g/ml}$ )	Control	Treated (20 $\mu\text{g/ml}$ )
A7436	$9.0 \times 10^6$	$2.0 \times 10^4$	$2.0 \times 10^6$	$6.8 \times 10^4$
MSM-1	$6.1 \times 10^6$	$6.4 \times 10^6$	$1.1 \times 10^7$	$1.1 \times 10^7$

characterization of a Tn4351-generated mutant of *P. gingivalis* A7436 that exhibits increased resistance to phagocytosis and killing by human PMNs has allowed us to begin to define putative virulence determinants responsible for the evasion of the host-mediated immune response.

The interaction between human PMNs and *P. gingivalis* has been an area of intense interest (5–7, 52, 53). Although *P. gingivalis* appears to evade the PMN response, the mechanisms involved in this process are not known. It has been speculated that capsular polysaccharide plays a role, but prior studies have been mostly correlative and have lacked genetically defined strains with which to work (53). The *P. gingivalis* polysaccharide-containing material appears to be composed of two antigenically distinct carbohydrate-containing antigens, as indicated by immunoelectrophoresis analysis (45); these may represent polysaccharide and LPS antigens. *P. gingivalis* strains have also been shown to express unique polysaccharide-containing immunodeterminants (45, 62), and successful opsonization of certain *P. gingivalis* strains may require antibody directed to these immunodeterminants to enhance complement activation and binding to the cell surface. The *P. gingivalis* MSM-1 polysaccharide-containing material appears to be antigenically distinct from that of *P. gingivalis* A7436, indicating that the insertion of Tn4351 into the *P. gingivalis* MSM-1 chromosome may have altered the production of this polysaccharide-containing antigen. The loss of the polysaccharide-containing antigen in *P. gingivalis* MSM-1 may result in enhanced production of LPS, with an overall increase in the quantity of total hydrophilic O-antigen, which may function to block antibody binding and also alter physical (surface tension) properties. The increased thickness of the ruthenium red-staining layer of *P. gingivalis* MSM-1 is consistent with this hypothesis.

Polysaccharide-encapsulated bacteria are the most successful in evading phagocytosis (1, 11, 19, 40, 51, 61), in part because of their hydrophilic nature. Studies with *P. gingivalis* also indicate that the more hydrophilic encapsulated strains are more resistant to phagocytosis than the more hydrophobic strains (52, 53). The presence of polysaccharide-containing capsule and LPS has been correlated with a reduction in complement activation and increased resistance to phagocytosis for several bacterial species (19, 21). Although both A7436 and MSM-1 were hydrophilic, MSM-1 was found to have increased hydrophilicity; the increased hydrophilicity of *P. gingivalis* MSM-1 may result in a decrease in the activation of complement, leading to decreased phagocytosis. Studies have shown (1, 60) that the hydrophobicity of encapsulated bacteria is enhanced by opsonization with antibodies to their surface components or by the addition of complement, giving rise to increased phagocytic ingestion *in vitro*. This could explain the results presented here; i.e., a significant difference in PMN phagocytosis of *P. gingivalis* MSM-1 was found when normal human serum was used. Contrastingly, in the presence of anti-A7436 or anti-MSM-1 serum, no differences were observed in PMN phagocytosis between *P. gingivalis* MSM-1 and A7436.

Surface polysaccharides may also restrict complement activation by preventing an interaction with activator molecules such as LPS, either by physically masking these molecules (19), by having an increased affinity for the regulatory molecules capable of inhibiting alternative complement activation (24), or by physically preventing the deposition of activated complement molecules via steric hindrance. Previous studies in *E. coli* have shown that LPS plays a vital role in preventing the attachment of antibody to the cell surface; smooth phenotypes that bind less antibody than rough strains do not activate the classical pathway of complement (42), resulting in decreased killing (21). In addition, *E. coli* mutants that are devoid of

O-antigen-containing capsule and contain 50% more LPS than the parent strain have been shown to be completely resistant to complement killing (13). Since the terminal membrane attack complex (C5b-9) must stably insert into a hydrophobic membrane site to effect killing, the increased coverage of LPS with O-antigen may preclude the access of C5b-9 to lethal sites on the cell surface. However, we do not believe the decreased killing observed for MSM-1 in this study is a result of decreased serum killing. Previous studies (6) suggest that *P. gingivalis* A7436 is resistant to serum killing but effectively killed by PMNs. Therefore, the lack of PMN killing of MSM-1 may be due to resistance to intracellular bactericidal enzymes. This hypothesis is supported by our studies with PMN granule extracts. Since we used a complex mixture of granule extracts to examine the resistance of *P. gingivalis* A7436 and MSM-1 to PMN antimicrobial activities, it is impossible to differentiate which antimicrobial mechanism could be operative.

The function of complement C3b and IgG in opsonization, the binding to PMN receptors, and the triggering of the respiratory burst and degranulation (and hence killing) are well-described phenomena (47). Recently, Edwards et al. (10), using monoclonal antibodies specific for complement receptor CR3 and complement receptor CR1, demonstrated that efficient PMN killing of encapsulated group B streptococci in nonimmune serum requires binding of both CR3 and CR1 receptors, while PMN killing of the unencapsulated strain was effective even with the CR1 and CR3 receptors blocked. The loss of K2 capsular antigen from *Klebsiella* spp. has also been shown to result in an increase in hydrophilicity and in susceptibility of this organism to PMN killing; moreover, this same K antigen appears to stimulate the release of lysozyme (a secondary granule enzyme) but not myeloperoxidase (a highly bactericidal, primary granule enzyme) (34). The effects of MSM-1 on primary and secondary granule release from PMNs are not known; however, one could speculate that MSM-1 has a reduced ability to stimulate the activation of PMNs, with a concomitant reduction in release of bactericidal enzymes, and hence ineffective PMN killing.

Several studies have indicated that the biochemical properties of *P. gingivalis* may also play a role in its immune evasion. Particularly significant may be the ability of *P. gingivalis* to degrade opsonic complement C3 (5, 43, 44). We have shown previously that *P. gingivalis* A7436 is fairly resistant to phagocytosis compared with several other *P. gingivalis* strains both *in vitro* and *in vivo* (6, 12). *P. gingivalis* A7436 fails to accumulate C3 on its surface and degrades C3 through potent constitutive protease activity; the enzymatic cleavage of C3 and IgG is inhibitable by opsonic hyperimmune antiserum or human convalescent-phase sera from adult periodontitis patients. It is unclear whether antibody directed solely to the polysaccharide antigens of *P. gingivalis* facilitates phagocytosis, as described for other bacterial species (10, 63). It is more likely that antibody directed to the proteases is primarily required to allow C3 deposition, while antibody directed to polysaccharide antigens may dictate the receptor-accessible localization of C3b and C3bi (46). These hypotheses are currently being investigated in our laboratory. We have not yet examined *P. gingivalis* MSM-1 for the ability to accumulate C3 on its surface; however, the ability of *P. gingivalis* MSM-1 to resist phagocytosis *in vitro* does not appear to result from an increase in the trypsin-like proteolytic activity (data not shown). In future studies, we will evaluate the ability of MSM-1 to activate complement and localize C3b on the cell surface.

In conclusion, we have isolated a mutant of *P. gingivalis* that exhibits an increased evasion of PMN-mediated phagocytosis and killing. Our initial studies suggest that the decreased hy-

drophobicity, the loss of an anionic polysaccharide antigen, and the overproduction of an O-side-chain polysaccharide-containing layer may be responsible for this observed resistance. In future studies, we hope to specifically identify the genetic lesion in *P. gingivalis* MSM-1 by cloning the corresponding wild-type gene from a genomic library prepared from the wild-type strain by using the Tn4351 insert in MSM-1 as a probe. These studies will allow us to begin to define putative determinants responsible for polysaccharide production and to correlate the expression of specific polysaccharide antigens with the pathogenic potential of *P. gingivalis*.

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