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The *rag* Locus of *Porphyromonas gingivalis* Contributes to Virulence in a Murine Model of Soft Tissue Destruction$^{\triangledown}$

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The *rag* locus of *Porphyromonas gingivalis* encodes a putative TonB-dependent outer membrane receptor, RagA, and a 55-kDa immunodominant antigen, RagB. Inactivation of either *ragA* or *ragB* prevented expression of both RagA and RagB. Both the *ragA* and *ragB* mutants were significantly less virulent than wild-type strains in a murine model of infection.

The *rag* locus of the periodontal pathogen *Porphyromonas gingivalis* encodes RagA, a 115-kDa outer membrane protein with features of a TonB-dependent receptor, and RagB, a 55-kDa antigen to which periodontal patients demonstrate an elevated immunoglobulin G response; together, these proteins are predicted to constitute a membrane transporter system (4, 8). Four variants of the *rag* locus have been detected among clinical isolates of *P. gingivalis* (7), and a significant association was observed between carriage of the *rag-1* allele and a highly virulent phenotype in a murine model of soft tissue destruction (7, 11). Indeed, in a variety of animal models of infection, *P. gingivalis* strains have been found to differ in their degrees of virulence, but *rag-1* strains W50 and W83 (for which the complete genome sequence has been reported [15]) are consistently reported to be among the most virulent (1, 6, 11, 14). *P. gingivalis* produces a number of well-characterized virulence factors, including proteases, fimbriae, and capsule (9). In order to determine whether the *rag* locus represents a further virulence factor for *P. gingivalis*, we have created insertion mutants with the *rag-1* genes and tested their effect in a murine model of soft tissue destruction. Since interstrain polymorphism had also been detected in PG0183, the gene upstream of *rag* (7), we additionally investigated the effect of mutation in this locus.

Genes were inactivated by insertion of the *erm* (*ermF-ermAM*) cassette from plasmid pVA2198, using an allele replacement strategy as described by Fletcher et al. (5). Briefly, the *erm* cassette (obtained by SacI plus PstI or SphI plus EcoRI digestion of pVA2198) was either inserted into the cloned genes (8) or ligated to PCR products to produce constructs in which the central region of each gene was replaced by the *erm* cassette. (PCR primers were as follows: for *ragA*, CGCTATTCTTCCTTTGCTTGCT and TTACCATCCGCATCGACTTGA; for *ragB*, AATACTGAAAATCCACGA and TAGGGGCTGCGACAAAAA; and for PG0183, GTGAACAAGCAGATTGGGG and CATAAGAGAGACGGAAACGAG). Prior to ligation with *erm*, the *ragA* product was digested with SacI plus PstI and the PG0183 product by SphI plus EcoRI. The DNA products were introduced into *P. gingivalis* by electroporation and transformants selected on media containing clindamycin, using methods described previously (16); replacement of the wild-type alleles was confirmed by demonstrating the expected length change for *ragA* or *ragB* by PCR and the expected restriction fragment changes by Southern blotting with *ragA* or *ragB* probes. In initial experiments, we were unsuccessful in the mutation of *ragA* or *ragB* in W50 but obtained mutants with mutations in both genes with the alternative *rag-1* strain WPH35 (provided by W. P. Holbrook [12]). Subsequently, both *ragA* and PG0183 were successfully inactivated in strain W50; we were unsuccessful in inactivating *ragB*, but this was not pursued since the *ragA* mutant was phenotypically *ragB* negative. It is unclear why the two strains differed with respect to our ability to inactivate *rag* genes. The mutant alleles generated are illustrated in Fig. 1. The expression of RagA and RagB was examined in mutants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2), with proteins prepared as described previously (4). A major 115-kDa outer membrane protein visible in both whole-cell and outer membrane protein profiles of W50 and WPH35 could not be detected after the disruption of *ragA.* The 55-kDa RagB protein was also absent in *ragA* mutants, as confirmed by Western blotting with the anti-RagB monoclonal antibody DRU55.5 (8, 12). (The same result as that shown in Fig. 2 was obtained for W50 Δ*ragA*::*erm* [not shown].) Similarly, both RagA and RagB were absent in the WPH35 *ragB* mutant. We have previously demonstrated that *ragA* and *ragB* are cotranscribed (8); it is not known whether the loss of both proteins when either gene is disrupted is due to a requirement for both proteins in order to maintain a stable outer membrane protein com-

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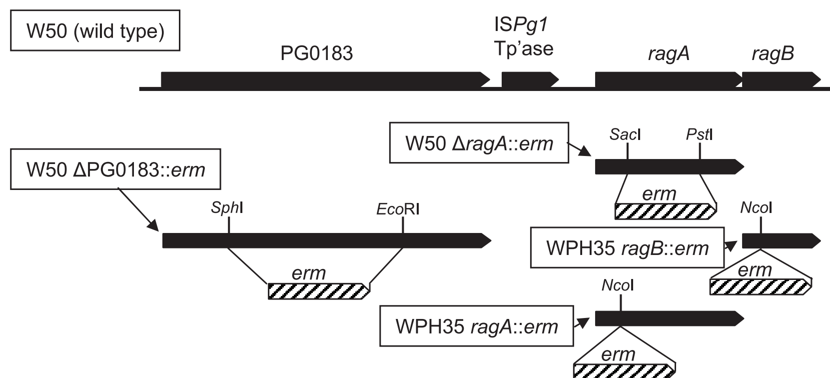

FIG. 1. Position of the *erm* (*ermF-ermAM*) cassette in *P. gingivalis* mutants. The upper line indicates the position of the *rag* operon and PG0183 in wild-type *P. gingivalis* W50 (8). Below are four mutant constructs in which the *erm* cassette is either inserted into, or replaces a section of, the corresponding genes of *P. gingivalis* W50 or WPH35. The positions of relevant restriction sites are shown.

plex or to a polar effect of insertion mutagenesis. Disruption of PG0183, a putative lipoprotein of unknown function encoded upstream of the *rag* locus, did not affect the expression of the Rag proteins.

The Rag proteins have sequence similarity to SusC and SusD, which mediate starch uptake in *Bacteroides thetaiotaomicron*, and also to proteins involved in iron uptake (7). *P. gingivalis* is asaccharolytic, but we nevertheless confirmed experimentally that supplementation of chemically defined medium (13) with maltose in the presence or absence of bovine serum albumin provided no detectable increase in the growth of *P. gingivalis* W50. The growth rate of *P. gingivalis* in brain heart infusion supplemented with hemin was unaffected by disruption of *ragA*, *ragB*, or PG0183. Similarly, there was no significant difference from the wild type in the ability of *ragA* or PG0183 mutants (the *ragB* mutant was not tested) to grow during repeated subcultures in the presence of hemin, hemoglobin, myoglobin, or lactoperoxidase as iron sources (2). Depletion of hemin by repeated subculture led to the suppression of growth at the same rate for mutants as for wild-type W50.

The virulence potential of the wild type and isogenic mutant strains of *P. gingivalis* was assessed in the murine model described by Kastelein et al. (10), by inoculation of bacteria at three different doses subcutaneously into the dorsolateral surfaces of eight mice per dosage group. A standardized

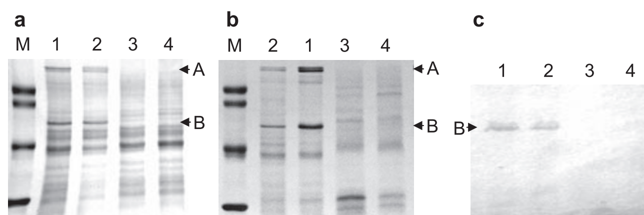

FIG. 2. Protein analysis of *P. gingivalis* W50 (lanes 1), WPH35 (lanes 2), WPH35 *ragA*::*erm* (lanes 3), and WPH35 *ragB*::*erm* (lanes 4), with molecular mass markers at 77, 66, 42, and 30 kDa (lanes M). (a) Whole-cell proteins stained with Coomassie blue; (b) outer membrane proteins stained with Coomassie blue; (c) Western blot of whole-cell proteins detected with *ragB*-specific monoclonal antibody DRU55.5 (12). Arrows labeled A and B mark the expected positions of RagA and RagB, respectively.

protocol, approved by the local ethics committee and the United Kingdom Home Office animal experimentation licensing authority, was applied as described previously (3). Animals were scored twice daily on the basis of appearance, body weight, and lesion size, and moribund animals or those with a lesion size of greater than 15 mm in any direction were sacrificed and recorded as deaths. Figure 3 illustrates Kaplan-Meier survival curves for experiments with three different doses of the WPH35- and W50-derived strains, respectively (experiments were replicated in full for WPH35 strains and very similar results obtained [not illustrated]). All mice inoculated with 2×10^{10} or 1×10^{10} CFU of wild-type WPH35 (in both replications of the experiment) had died or been euthanized by 2.5 days (as shown in Fig. 3a). By contrast, all or seven of eight animals inoculated with the same inocula of WPH35 *ragA*::*erm* survived to the end of the study, though localized lesions, which resolved and healed, were observed. (In initial experiments, animals were followed for up to 15 days, but no further deaths occurred after 3 days, so later experiments were truncated after 4 days.) The *ragB* mutant of WPH35 appeared somewhat less attenuated, as the highest inoculum resulted in the death of the majority of mice; nevertheless, at the lower inocula, all mice receiving WPH35 *ragB*::*erm* survived. Similarly, all mice inoculated with wild-type W50 at all three dosages had died or been destroyed by 24 h, whereas with inocula of 1×10^{10} and 5×10^9 CFU per mouse of W50 Δ*ragA*::*erm*, only one death occurred in each group within 24 h and the majority of animals survived (Fig. 3b). The highest inoculum of W50 Δ*ragA*::*erm* resulted in the death of all eight mice within 48 h. In all experiments, the survival of both *ragA* mutant- and *ragB* mutant-inoculated mice was significantly greater ($P < 0.01$, log rank test, conducted with GraphPad Prism software) than that of mice inoculated with the isogenic wild-type strains at all bacterial loads tested. At the highest inoculum only, the survival of mice inoculated with WPH35 *ragA*::*erm* was significantly greater than that of those inoculated with WPH35 *ragB*::*erm*. A minority of animals inoculated with W50 ΔPG0183::*erm* survived longer than those inoculated with wild-type W50, but the increased survival was not statistically significant in these experiments.

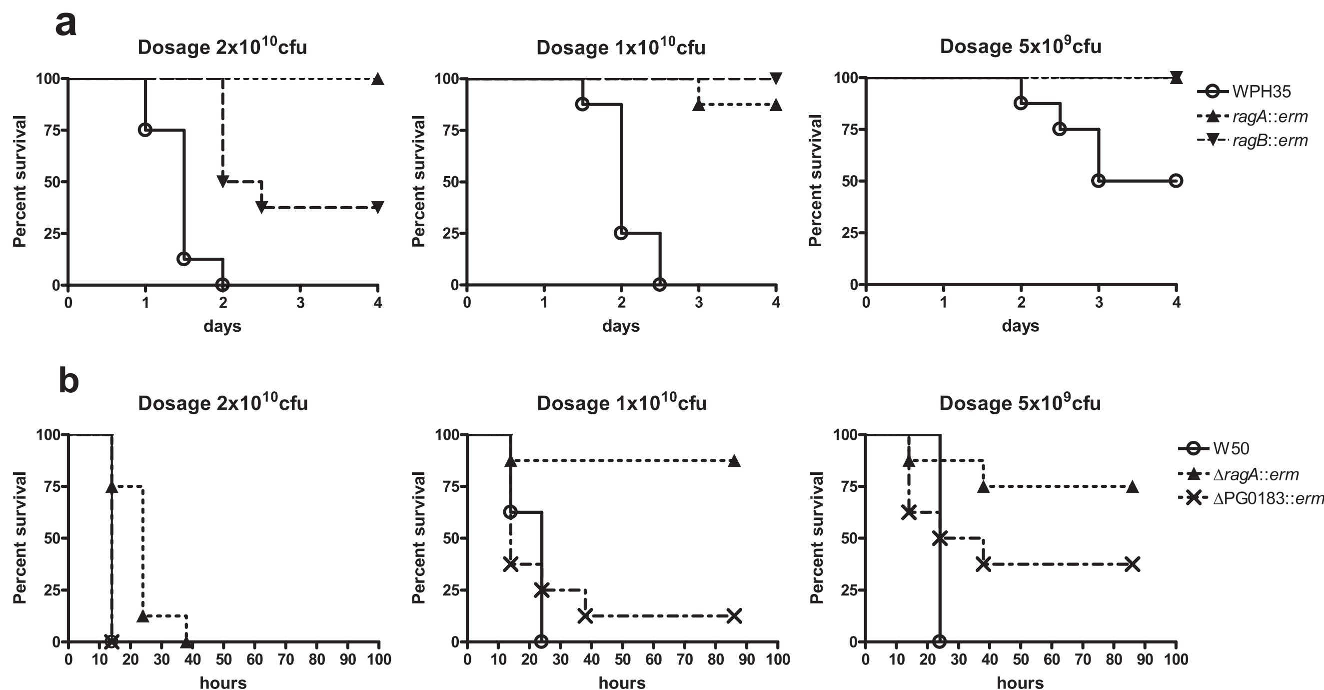

FIG. 3. Kaplan-Meier survival curves for mice inoculated with wild-type and mutant *P. gingivalis* at different doses (numbers of CFU per mouse). (a and b) Two separate experiments. Eight mice were inoculated with each *P. gingivalis* strain at each dosage. In each experiment, all mice were inoculated on the same day with a single preparation of each strain, appropriately diluted.

The results show clearly that inactivation of the *rag* locus reduced the virulence of *P. gingivalis* in a mouse model of soft tissue destruction. This represents one of very few practical models for *P. gingivalis* and is widely used for virulence studies despite some uncertainty about the interpretation of results with respect to periodontal disease. The effect on virulence was not due to a general growth defect of *rag* mutants, since growth in vitro was not changed; however, we cannot rule out the possibility of an impairment to growth in vivo. The *ragB* mutant was somewhat less attenuated, despite the apparent loss of both the RagA and RagB proteins in outer membrane preparations and in whole-cell lysates (Fig. 2). We speculate that the residual expression of RagA and possibly a truncated form of RagB may be sufficient to retain a low level of activity. The function of the Rag proteins remains unclear, but their similarity to membrane transporters, and in particular to numerous loci of *Bacteroides thetaiotaomicron* strongly implicated in the uptake of diverse dietary polysaccharides, suggests a role in acquiring a macromolecule from the external milieu. Given the contribution of the *rag* locus to virulence described here, this might involve a macromolecule that aids survival or dissemination within the host but is not required in culture media.

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