The rag Locus of Porphyromonas gingivalis Contributes to Virulence in a Murine Model of Soft Tissue Destruction

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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 locus (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 has also been detected in PG0183, the gene upstream of rag-1 (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, CGCTATTCTTCTTTGTGCT and TTACCATCGGATCGACTTGA; for ragB, AATACTGAAAT CCAAGA and TAGGGGCTGGGACAAAAA; and for PG0183, GTGAACAGACAGATTGGG and CATAAGA GAGACGAAACGAG). 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 (10); replacement of the wild-type alleles was confirmed by demonstrating 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 ΔragA::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-
In brain heart infusion supplemented with hemin was unaffected by disruption of ability of P. gingivalis. The growth of bovine serum albumin provided no detectable increase in medium (13) with maltose in the presence or absence of experimentally that supplementation of chemically defined medium (13) with maltose in the presence or absence of iron sources (2). Depletion of hemin by repeated subculture of hemin, hemoglobin, myoglobin, or lactoperoxidase as tested) to grow during repeated subcultures in the presence or absence of 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 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).

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 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 ΔragA::erm, only one death occurred in each group within 24 h and the majority of animals survived (Fig. 3b). The highest inoculum of W50 ΔragA::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.
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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