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Critical pathways in microbial virulence

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Abstract

The virulence of a microbe represents a combination of complex factors including the agent's transmissibility and the severity of the disease associated with infection and is also significantly influenced by the susceptibility of the colonized host. Virulence factors may be defined as those products of the organism which are required to complete the various stages of the life cycle leading to pathology in the host. In this review, we examine some of the approaches which have been adopted in other fields of infectious disease in order to categorically identify virulence factors using a classical genetics approach with relevant models or human subjects. The absence of an accurate experimental model for periodontal disease means that our understanding of the microbial virulence determinants and pathways in this disease remains hypothetical and based largely on observations in vitro. However, factors which enable the organism to persist in spite of the elevated immune and inflammatory pressure at sites of disease are liable to be critical. Periodontal bacterial genomics is liable to make a significant impact on the field through an increased appreciation of the role of gene acquisition and gene loss in the evolution of periodontal bacteria and of the consequences of strain variation in gene content on virulence potential.

Key words: gene expression; pathogenicity; virulence

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The virulence of a microbial pathogen is generally defined as the degree of pathogenicity or ability of the organism to cause disease measured by an experimental procedure. It represents a combination of highly complex parameters and depends upon both the relative infectivity of the organism and the severity of the disease produced. However, in all cases these two parameters of infectivity and disease severity are profoundly influenced by the nature and status of the host organism or the site of colonization in that host. Thus, a breach in the normal defensive barriers of the host, through, for example, trauma, immunosuppression/dysfunction or co-infection by another organism, can dramatically increase the virulence of a given organism. Hence, any description of microbial virulence is fundamentally reliant on an understanding of the relative susceptibility of the colonized host.

The accepted definitions of a microbial pathogen and microbial virulence have been formulated largely from the study of infections caused by a single aetiological agent and build upon the meticulous and rigorous investigations of Robert Koch on diseases including anthrax and tuberculosis. Koch's resultant postulates were an essential guide to identifying the specific causation of infectious diseases:

1. The parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease.
2. The parasite occurs in no other disease as a fortuitous and non-pathogenic parasite.
3. After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the diseases anew.

In more recent times, the definition of a pathogen has been amended and in some senses broadened to ''an organism which reproduces and persists on or within another species by breaching or destroying a cellular or humoral host barrier'' (Falkow 1988).

The virulence determinants of a pathogen can simply be defined as those gene products which facilitate colonization, growth and survival within the diseased host organism and spread to a new host. The adaptation of Koch's postulates by Falkow (1988) to experimentally define the genes involved in virulence (the Molecular Koch's postulates) now serve as the template for a contemporary and similarly experimentally rigorous approach to the identification of the molecular basis of microbial virulence. These adapted postulates include:

1. The phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species.

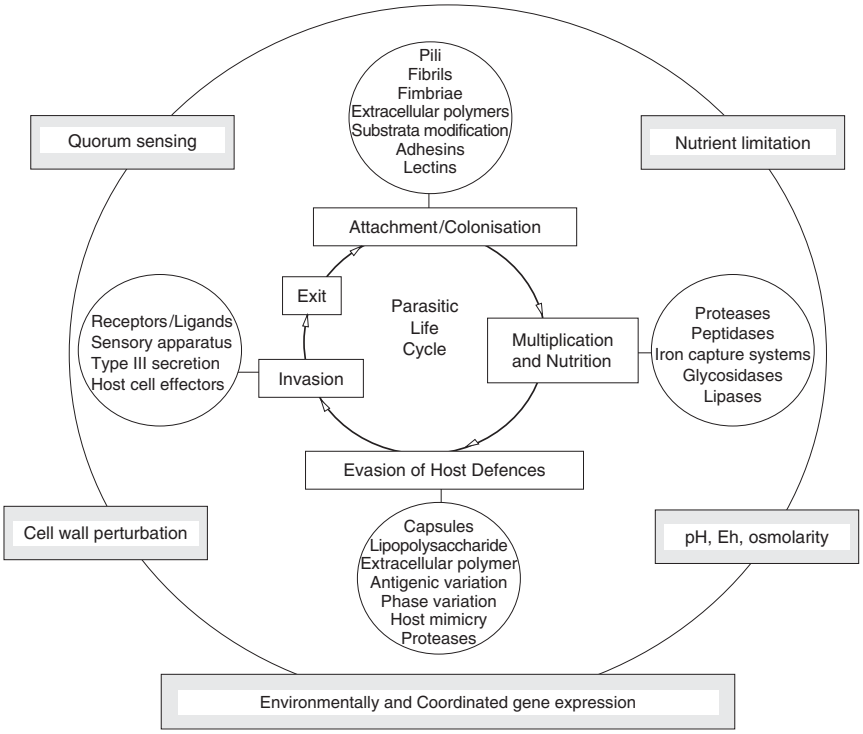

Fig. 1. Stages in the life cycle of a parasite and influences on the genetic regulation of these processes

2. Specific inactivation of the gene(s) associated with the suspected virulence trait should lead to a measurable loss in pathogenicity or virulence, or the genes associated with the supposed virulence trait should be isolated by molecular methods.
3. Reversion or allelic replacement of the mutated gene should lead to restoration of pathogenicity.

Established Examples of Critical Determinants of Microbial Virulence

The requisite stages in the life cycle and spread of one organism which parasitizes another are presented in Fig. 1. The key steps are: initial colonization and attachment; multiplication and nutrition; evasion of the host defences; (in some cases) invasion and, lastly, exit in order to disseminate to a new host. Specific gene products (presumptive virulence factors) are required to facilitate each of these processes, and these products will vary from organism to organism dependent upon the particular strategy employed to satisfy each element of the life cycle. The gene products or traits associated with each step in the life cycle presented in Fig. 1 represent examples drawn from multiple organisms. Disease can be defined as the unfavourable outcome to the host by the application of these life cycle stages of the pathogen in a *susceptible host background*.

While there are multiple examples of the use of a classical genetics approach to fulfil Koch's molecular postulates to define critical virulence determinants, shown in Fig. 1, studies on the role of P-pili of uropathogenic *Escherichia coli* (UPEC) in attachment/colonization deserve particular mention. P-pili are composite organelles consisting of a short, flexible-tip fibrillum attached to the distal end of a thicker rod structure. The tip fibrillum, which is about 2 nm thick, contains a distally located adhesin, PapG, which recognizes glycolipid receptors expressed by erythrocytes and host cells present in the kidney (Mulvey et al. 2001). P-pili, and specifically the PapG adhesin, are significant virulence factors associated with pyelonephritis. For example, Roberts et al. (1994) demonstrated that PapG, while unnecessary for UPEC colonization of the bladder, is essential for a pyelonephritic UPEC isolate to adhere to renal tissue and cause pyelonephritis in cynomolgus monkeys. In this investigation, the virulence of a pyelonephritic strain DS17 which expresses P-pili was compared with a mutant strain, DS17-8, which expresses P-pili lacking the PapG adhesion. In cynomolgus monkeys, DS17 and DS17-8 were equally able to cause bladder infection, whereas only the wild-type strain DS17 could cause pyelonephritis. As DS17, but not DS17-8, adheres to renal tissue, these data underscore the critical role of microbial adherence to specific host tissues in infectious disease and strongly suggest that the PapG tip adhesin is essential in the pathogenesis of human kidney infection. Similarly, Wullt et al. (2000) demonstrated, using human volunteers, that P-pili enhance the colonization of the urinary tract and facilitate the establishment of bacteriuria by an UPEC isolate known to cause asymptomatic bacteriuria. Patients were subjected to intra-vesical inoculation with an asymptomatic bacteriuria strain, *E. coli* 83972, or its P-piliated transformants. The transformants established bacteriuria more rapidly than *E. coli* 83972, required a lower number of inoculations to reach 10^5 cfu/ml, and bacterial establishment was shown to vary with the in vivo expression of P-pili in these patients.

While the interpretation of these studies benefits enormously from the use of a natural human or primate model of the disease, significant advancement of our identification and understanding of virulence determinants has been derived from in vivo models in alternative systems, particularly for broad host range pathogens. For example, in the case of the environmental, opportunistic pathogen *Pseudomonas aeruginosa*, bacterial pathogenicity models employing plant and non-mammalian animal hosts have been developed (Mahajan-Miklos et al. 2000). Such models rely on common parallels in innate immunity and conservation of the molecular basis of bacterial pathogenesis in evolutionary divergent hosts. The Type III secretion system (TTSS) of *P. aeruginosa* has been investigated in such a manner. TTSS mediate the delivery of toxins directly into host eukaryotic cells and are well established as major virulence factors in the pathogenesis of *Yersinia*, *Shigella*, *Salmonella*, *E. coli* and *Chlamydia* infections (Hueck 1998) in which they facilitate processes of attachment and invasion. *P. aeruginosa* is a major opportunistic pathogen causing nosocomial pneumonia, burn wound infections, corneal ulceration in contact lens wearers, septicaemia in the immunocompromised and respiratory exacerbations in patients with cystic fibrosis. It contains a

TTSS homologous to the *Yop* virulon of *Yersinia enterocolitica* (Salyers & Whitt 1998) which encodes at least four effector (exoproduct) molecules, exoenzymes (Exo) S, T, U and Y, and is required for the full expression of virulence in a variety of model systems. The TTSS of two invasive strains, PA01 and CHA, was recently investigated using *Drosophila melanogaster* as a host. Isogenic mutants in *ExsA*, a positive regulator of the TTSS, were shown to be less virulent in this model, whereas rapid killing involved activation of the TTSS genes at the transcriptional level (Fauvarque et al. 2002). In the non-invasive cytotoxic isolate PA103, ExoU and the TTSS were shown to be important in a *Dictyostelium discoideum* model of pathogenicity. *D. discoideum* was able to feed on mutants lacking a functional TTSS or *exoU* gene, whereas those fed on wild-type PA103 were rapidly killed. The importance of the *P. aeruginosa* TTSS to human infection is supported by the finding that aggressive infection and higher mortality is correlated with the ability to secrete TTSS toxins in vitro (Roy-Burman et al. 2001).

Superimposed upon the complement of genes required for each step in the life cycle of a given organism are the regulatory mechanisms which allow for the co-ordinated expression of these gene products at an appropriate stage in the infection process and/or in response to appropriate environmental cues. In some instances, the gene products involved in these regulatory functions can also be described as virulence determinants. For example, two-component regulatory systems control gene expression in many bacteria in response to environmental signals. They are composed of a membrane-associated sensor kinase protein and a cytoplasmic transcriptional regulator. In response to external stimuli, the membrane-located sensor undergoes a conformational change which results in phosphorylation of the regulator. This in turn affects its ability to bind to DNA at specific promoter sites and thus regulates its activity as a transcriptional regulator. Many two-component systems are involved in controlling virulence gene expression in response to changes in magnesium levels and pH. For example, the *Salmonella* PhoP–PhoQ response regulator system regulates more than 40 different genes which affect survival within macrophages, resistance to host antimicrobial peptides and acid pH and invasion of epithelial cells. Inactivation of the PhoP–PhoQ system attenuates virulence by more than 10,000-fold in mouse model systems (Miller et al. 1989, 1993).

Virulence of Periodontal Bacteria Assessed using the Classical Approach

The classical genetic approach to identifying critical determinants of virulence described in the previous sections is fraught with difficulties when these same principles are applied to periodontal organisms. There are several well-rehearsed reasons. First, although it is acknowledged that the microbial challenge is a key determinant in the initiation and progression of the disease, this is a complex bacterial microbiota, only a proportion of which is cultivatable and only a tiny fraction of which has been characterized at the cultural and biochemical level. Second, the definition of a periodontal pathogen relies very heavily on quantitative differences in the numbers of a given organism in healthy *versus* diseased individuals (and/or affected *versus* unaffected sites in the same individual), or, in some cases, on the assessment of the previous interaction of bacteria with the immune system of patients and controls via analysis of the antibody response. Therefore, Koch's second postulate (that the parasite occurs in no other condition as a fortuitous and non-pathogenic parasite) remains frequently unproven. For both these reasons, the determination of which organism, or more precisely, which combination of organisms are critical to the disease process remains controversial, although there are clearly several very strong individual contenders or consortia (Socransky et al. 1998). Third, even when a presumptive aetiological agent is identified through a combination of microbiological and immune investigations and in vitro characterization, the experimental methodology for the identification and verification of virulence determinants is fundamentally dependent upon a practical and relevant model system. In the case of periodontal disease, there is no experimentally practical model which accurately mimics the pathogenesis, both in terms of the diversity of the microbial challenge and in terms of the chronic natural history of the disease. This is regrettable because the absence of these systems not only prevents confirmation of the role of candidate virulence determinants in the disease process, but also the use of novel molecular methodologies such as signature tagged mutagenesis to identify new virulence determinants (Unsworth & Holden 2000). Animal models in current use include: estimation of soft-tissue necrosis and/or lethality in a mouse following injection of live bacteria, either subcutaneously or into chambers implanted subcutaneously, rodent oral colonization models and rodent oral bone loss models. In most cases, the challenge involves monoinfection with large doses of the candidate pathogen, and, almost exclusively, the pathology arising from these procedures is acute in nature and bears little resemblance to the actual natural history of the disease in humans. Some examples of how these models have been applied to periodontal organisms with mutations in candidate virulence genes are presented in Table 1. Although not a comprehensive list, overwhelmingly the studies have focused upon *Porphyromonas gingivalis* and have largely concentrated upon the effect of loss of function of proteolytic enzymes in the mouse lesion model.

Increasingly, elegantly designed in vitro-based methodologies are being employed to examine the bacterial products expressed uniquely in vivo by periodontal organisms (Wu et al. 2002). These investigations are beginning to shed light on which gene products are present during the infection process and the potential mechanisms of, for example, invasion into epithelial cells, deregulation of immune cell function and pro-inflammatory or immunomodulatory action of either whole bacteria or their purified products. While these are valuable data, frequently showing parallels with other more well-characterized host–pathogen interactions, they inevitably fall short of providing the in vivo relevance of the investigated phenotype/pathogenic trait to the natural history of the disease. Only in very rare instances has the virulence trait of a periodontal organism been experimentally manipulated in a human population to determine the impact on either colonization or disease. One example is the use of passive immunization of the cleaned tooth surfaces of periodontal patients with a monoclonal antibody to an adhesion domain on the RgpA protease of *P. gingivalis*. In this instance, passive

Table 1. In vivo assessment of gene function in periodontal bacteria using isogenic mutants

Organism, strain	Gene	Putative function	Model of virulence, outcome*	Reference
Actinobacillus actinomycetemcomitans CU1000N	*tad*, *flp-1*	Tight adherence, bundled fibrils formation	Rat (oral administration, colonization), no bone loss, poor humoral immune response	Schreiner et al. (2003)
Porphyromonas gingivalis W83	*rgpA*	Arg-gingipain protease	Mouse (death), attenuated	Fletcher et al. (1995)
P. gingivalis W50	*kgp*, *rgpAB*	Lys-gingipain protease	Mouse (death), attenuated; *kgp* > *rgpAB*	Curtis et al. (2002)
P. gingivalis W83	*kgp*	Lys-gingipain protease	Mouse (death, lesion), attenuated	Lewis & Macrina (1998)
P. gingivalis ATCC33277	*rgpA*, *rgpB*, *kgp*	Arg- and Lys-gingipains	Mouse (lesion formation) diminished lesion formation	Yoneda et al. (2001)
P. gingivalis W50	*rgpA*, *rgpB*, *kgp*	Arg- and Lys-gingipain proteases	Mouse (death), attenuated: *kgp* > *rgpB* ⩾ *rgpA*	O'Brien-Simpson et al. (2001)
P. gingivalis W83	*dpp*	Dipeptidyl peptidase IV	Mouse (death), attenuated†	Kumagai et al. (2000, 2003)
P. gingivalis W83	*met*	L-methionine-α-deamino-γ-mercaptomethane-lyase	Mouse (death), attenuated	Nakano et al. (2002)
P. gingivalis W50	*luxS*	Quorum sensing	Mouse (death), no change	Burgess et al. (2002)
P. gingivalis ATCC33277	*fimA*, *mfa1*	Major and minor fimbrillin	Rat (alveolar bone loss), significant loss Double > *fimA* > *mfa1*	Umemoto & Hamada (2003)

*Outcome corresponds to the observation using the mutant relative to wild type.
†Intermediate.

immunization resulted in a significant reduction in re-colonization by this organism compared with sham immunized controls, suggesting that this determinant is indeed an important virulence factor for *P. gingivalis* (Booth et al. 1996).

Alternative Approaches to Defining Virulence Determinants in Periodontal Bacteria

It should be evident from the preceding sections that the classical genetic approach has significant drawbacks to the determination of critical pathways in microbial virulence in periodontal disease. Hence, alternative approaches are required and have been employed which draw upon both the experience of research in virulence mechanisms in other settings and, importantly, our theoretical understanding of the pathogenesis of periodontal disease. The scheme of molecular interactions in this disease as described by Kornman and Page (Kornman et al. 1997) is outlined below.

The hard, non-shedding surfaces of the teeth provide a unique site in the human body for colonization by a distinct bacterial microflora. Estimates of the number of different species which have evolved to inhabit this niche vary, but recent molecular analyses via sequence analysis of cloned 16S RNA genes, suggest that it is in excess of 600 taxa, the majority of which are found at no other mucosal location (Paster et al. 2001). In most individuals, the juxtaposition of these complex biofilms against the adjacent soft tissues of the periodontium, has only minor pathological consequences. A dynamic balance exists between the bacteria and their extracellular products and the host response, which is largely mediated through the action of polymorphonuclear leucocytes and their associated opsonins. The resultant mild inflammation, or gingivitis, is both non-destructive and easily reversible through mechanical removal of the bacterial deposits from the supra- and subgingival surfaces.

However, in a proportion of the population, this controlled, harmonious interaction is replaced by a persistent and aggressive relationship in which both the microbial challenge and the host's inflammatory response are intensified (Kornman et al. 1997). This scenario is characterized by the following

hallmarks. First, the composition of the cultivatable bacterial flora changes from that found supragingivally, to a complex and predominantly anaerobic population, many of which utilize an asaccharolytic metabolism fuelled by the production of extracellular hydrolytic enzymes. Second, to counter this challenge, the magnitude of the host response, in the form of a plasma exudate and migrating phagocytic cells, is increased. Third, the epithelial attachment to the surface of the tooth migrates down the apical axis leading to the formation of deep periodontal pockets. Fourth, the deregulated interaction between the microbial challenge and the host response impinges upon the integrity of the epithelial boundary and underlying soft connective tissue and bone, largely through the direct action of inflammatory cell enzymes and activation of the underlying stromal cells. The net result is an irreversible destructive (Johnson et al. 1988) condition ultimately characterized by loss of the bony attachment apparatus and tooth loss (Curtis 2003). Finally, and critically in the context of this review, the altered environmental conditions favour the survival of anaerobic species and those equipped to survive the elevated inflammatory onslaught (Marsh 1994).

Hence, a key feature of the bacteria driving this chronic process is their ability to withstand the killing and clearance mechanisms of the host for a prolonged period despite colonizing an ecological niche under intense inflammatory pressure. This period may extend to decades for affected individuals. Destructive periodontal disease can thus be viewed as a major example of the family of chronic bacterial diseases which afflict the mucosal surfaces of humans. This family also includes inflammatory diseases of the gastrointestinal tract (e.g. inflammatory bowel disease and *Helicobacter pylori*-associated gastritis and gastric cancer), of the genitourinary tract (e.g. bacterial vaginosis) and persistent infectious diseases of the genetically or immunocompromised, *P. aeruginosa* and *Burkholderia cepacia* infections of the cystic fibrosis lung and *Mycobacterium tuberculosis* infection of HIV-infected individuals.

From this it follows that examination of the properties of periodontal bacteria which enable them to resist the antimicrobial properties of an elevated inflammatory and immune response may provide some clues to the elusive critical pathways of virulence in this disease. These properties may be divided into a number of functionally distinct categories and examples of each class are presented below – drawn largely from the extensive literature on *P. gingivalis*.

Protective Coats

Polysaccharides at the surface of a bacterium have important functions in the maintenance of the structural integrity of the cell and protection from external injury. In addition, as they constitute the boundary of the cell with the surrounding medium, they are central to recognition and molecular dialogue with the environment. Gram-negative bacteria are guarded from their environment by the outer membrane, which contains the unique glycolipid, lipopolysaccharide (LPS) and in some instances by a capsular polysaccharide. The O-antigen of LPS, a repeating unit polysaccharide of variable chain length, is important for the evasion of complement, particularly by the alternative cascade, and may also provide resistance to cationic antimicrobial peptides. Modifications to LPS structure, through, for example, the removal or decoration of the lipid A phosphates and fatty acyl chains, and the addition of 4-amino-4-deoxy-L-arabinose or phosphoethanolamine can further influence resistance to host antimicrobial factors or immune system recognition.

Bacterial capsules have long been accepted as important virulence determinants and, in invasive infections, the interaction between capsule and the host's immune system may decide the outcome of infection (Moxon & Kroll 1990). One of the most striking features of capsules is their diversity. For example, both *Streptococcus pneumoniae* and *E. coli* express over 80 chemically and serologically distinct capsules, and a single strain of *Bacteroides fragilis* is capable of the expression of multiple capsular types (Krinos et al. 2001). Typically they are composed of hydrophilic, negatively charged polysaccharides which help resist desiccation, complement mediated lysis and phagocytosis. Differences in virulence between capsular types of the same species are evident, for example, the K1 and K5 polysaccharides of *E. coli* camouflage the bacteria because of the similarity with host tissues.

Six different capsular serotypes have been described for *P. gingivalis*, although to date there is no structural information for any, save for gross compositional data of K1 (Farquharson et al. 2000). The presence of capsule has also been correlated with virulence of *P. gingivalis* using in vivo model systems. In one study, all encapsulated strains caused a spreading type of infection in a murine model accompanied by exudation, necrosis, ulceration and recovery of encapsulated bacteria systemically. Conversely, all un-encapsulated strains induced only localized abscess formation (Laine et al. 1997b, Laine & van Winkelhoff 1998). Epidemiological studies are limited. However, all serotype K antigen strains were represented in one periodontal study population (Laine et al. 1997a), and another study of the immunoglobulin (Ig)G antibody response reported that *P. gingivalis* types expressing either K1 or K6 may be more likely to participate in periodontal infections than others (Sims et al. 2001). In summary, while it is evident that the presence of capsule and the capsular type are liable to be important determinants of *P. gingivalis* pathogenicity, their contribution to the well-documented variations in virulence in this organism and their association with disease severity are unknown.

Full Frontal Assault

This strategy involves an offensive rather than defensive approach to the avoidance of the antimicrobial activities of the host. Examples from the periodontal literature include the well-characterized leucotoxin of *Actinobacillus actinomycetemcomitans* which may specifically disable phagocytic cell function in the periodontium (Ebersole et al. 1990) and the more recently investigated cytolethal distending toxin of this organism (Sugai et al. 1998). Proteolytic enzymes also fall within this category. While these activities may be important for the provision of microbial carbon, nitrogen and micronutrient sources, their frequently observed ability to interact with components of the host defences in a deleterious manner makes them attractive putative virulence factors.

P. gingivalis produces several extracellular proteolytic enzyme activities

with differing peptide bond specificities which have a number of in vitro properties consistent with a role in the periodontal disease process. Among these properties are deregulatory/destructive effects on components of the host defences operative in the periodontium. A more detailed review of this area is presented elsewhere (Curtis et al. 2001) but some of the key points are given here.

It has been recognized for some time that *P. gingivalis* cells trigger activation of the complement cascade of human plasma but fail to accumulate C3-derived opsonins on the cell surface. The link with proteolytic activities of the organisms was initially established by pre-treatment of whole cells with protease inhibitors prior to exposure to complement, which then allowed C3b and iC3b deposition (Schenkein 1989). In addition, it has been proposed that although opsonophagocytosis of *P. gingivalis* by human neutrophils is dependent upon a specific IgG antibody, the opsonization proceeds partly as a result of IgG antibody-dependent alternative complement, pathway activation (Cutler et al. 1993). The implication of these findings was that the specific antibody in immune sera may contain anti-*P. gingivalis* protease activity which prevents interference with the complement cascade. More definitive evidence that the cysteine proteases of *P. gingivalis* play a crucial role in the evasion of complement has come from studies using a *rgpA* mutant of *P. gingivalis* W83. In comparison with the parent strain, the mutant is less able to degrade purified C3, accumulates greater amounts of C3-derived opsonins on the cell surface in the presence of normal human serum and is phagocytosed by human neutrophils more efficiently (Schenkein et al. 1995).

The mechanism of complement inactivation by *P. gingivalis* has been established using purified preparations of C3 and C5 (Wingrove et al. 1992). $RgpA_{cat}$ was shown to perform a stepwise degradation of these two critical complement factors. C3 is initially cleaved to generate a C3a-like molecule and a C3b-like fragment followed by further degradation of the C3a component. C5 is consecutively cleaved at two sites in the α chain with the resultant generation of a small peptide which possesses potent neutrophil chemotactic properties analogous to C5a. As these proteolytic conversions can take place in the fluid phase, the resultant activation of complement can proceed in the absence of bacterial cell surface deposition. What is more, it is suggested that the concomitant production of a C5a-like neutrophil chemoattractant may have a significant effect on the inflammatory status at sites harbouring this bacterial enzyme activity. Less information is available concerning the degradation of Ig's by *P. gingivalis* proteases, although IgA, IgG and IgM are all susceptible to cleavage (Kilian 1981, Sundqvist et al. 1985, Sato et al. 1987).

Perturbation of neutrophil function by *P. gingivalis* proteases is not confined to effects on opsonins as there is accumulating evidence that these enzymes may also have direct effects on the phagocytic cells. For example, Kgp and a non-cysteinyl protease of *P. gingivalis* are able to cleave the C5a receptor on human neutrophils (Jagels et al. 1996a, b). Furthermore, purified preparations of $RgpA_{cat}$ and Kgp have been shown to interfere with the respiratory burst of neutrophils leading to a reduction in the generation of active oxygen species (Kadowaki et al. 1994, Abe et al. 1998) and these effects are abrogated in protease mutants (Nakayama et al. 1995).

A further area of subversion of the innate host defences relates to the action of proteases on antimicrobial peptides which are produced by neutrophils and epithelial cells and which are also present in the salivary secretions. These highly cationic molecules represent an ancient and very highly conserved component of the innate host defences. Because they frequently contain a large number of arginine or lysine containing peptide bonds, they would appear to be potential substrates for the Rgps and Kgp of *P. gingivalis*. This was demonstrated by Devine et al. (1999), who showed that these enzymes are able to proteolytically inactivate members of several antimicrobial peptide families. Conversely, however, *P. gingivalis* was still susceptible to killing by these compounds suggesting that, at least under in vitro conditions, the rate of degradation of these peptides is insufficient to protect the cell from their antimicrobial action. Nonetheless, the action of the Rgps and Kgp on antimicrobial peptides may contribute to a general diminution of innate defences in the subgingival region.

The maintenance of tissue homeostasis and the orchestration of the innate defences and specific immune response are dependent upon an inter-cellular communication network mediated through cytokines.

The influence of the cysteine proteases of *P. gingivalis* on the integrity and function of the cytokine network has been addressed by a number of authors and degradation of tumour necrosis factor-α, interleukin (IL)-6, and IL-1 and modification of IL-8 by the Rgps and Kgp have been demonstrated (Calkins et al. 1998, Mikolajczyk-Pawlinska et al. 1998, Sharp et al. 1998, Banbula et al. 1999). While these studies suggest that the Rgps and Kgp could have a catastrophic effect on the cytokine network in the periodontal tissues through degradative effects, it is also clear that components of *P. gingivalis* can stimulate the production of these key mediators of the inflammatory response (Lamont & Jenkinson 1998). Indeed, while the study of Sharp et al. (1998) demonstrated that HRgpA is able to functionally inactivate IL-1, these authors also demonstrated that an internal peptide derived from RgpA is a potent stimulator of IL-6 synthesis by human fibroblasts and mononuclear cells. The conflict between up-regulation of synthesis and proteolytic degradation of cytokines complicates the interpretation of the true biological significance of proteolysis in this system. However, as with other aspects of the interaction of the proteases of this organism with host components, a deregulated system is the most likely outcome.

Invasion of Host Cell as Means of Evasion of Extracellular Immunity

One suggested mechanism, a component of the so-called ''stealth technology'' practiced by some periodontal organisms including *P. gingivalis* and *A. actinomycetemcomitans*, involves entry into other host cells, primarily epithelial cells, to gain access to an immune privileged site (Lamont & Jenkinson 1998, Fives-Taylor et al. 1999). Verification of this process in vivo is now emerging by the detection of these and other species using fluorescent labelling within buccal epithelial cells taken directly from the mouth (Rudney et al. 2005). In the case of *P. gingivalis*, the organism has been shown to rapidly invade epithelial cells derived from the human gingiva and accumulate and persist in high numbers with a perinuclear localization (Lamont & Jenkinson 2000).

This positioning is similar to the localization observed for purified preparations of RgpA which is able to translocate the plasma membrane of epithelial cells (Scragg et al. 2002). While the precise mechanism is still under investigation, FimA, a major fimbriae and the gingipain proteinases are required for the attachment and internalization of the bacterial cells. In the case of *A. actinomycetemcomitans*, while the precise details of the mechanism are unknown, there is a suggestion that the invasion process may be augmented by soluble CD14 derived from saliva (Takayama et al. 2003).

Manipulation of the Host Response

It is becoming increasingly evident that microbial organisms, having co-evolved with the innate defence systems of their respective hosts, have developed strategies not only to overcome protective host barriers but also to manipulate these systems to their own advantage. One example of this phenomenon is the ability of cell surface proteins of both Gram-negative and Gram-positive bacteria, including *A. actinomycetemcomitans* and *P. gingivalis*, to influence the pattern of cytokine expression by host cells. The term ''bacterial modulins'' was introduced by Henderson et al. (1996) to describe these bacterial cytokine-inducing molecules because of their ability to modulate eukaryotic cell behaviour (Henderson et al. 1996). More recently, a sophisticated manipulation of the host response by *P. gingivalis* has been described as a consequence of the biological properties of different molecular species of the lipid A portion of the LPS of this bacterium (Darveau et al. 2004). Some of these lipid A species are able to act as agonists of the host response through Toll-like receptor signalling, and thus have similar biological properties to the hexa-acylated lipid A species of enteric organisms. Conversely, other lipid A moieties produced by *P. gingivalis* act as antagonists of this signalling pathway and are able to block the activity of the pro-inflammatory lipid A forms. This has led to the suggestion that by altering the proportions of the different lipid A components, *P. gingivalis* may be able to manipulate the innate response in order, for example, to down-regulate the inflammatory response as a defensive measure.

The Impact of Genomics on the Characterization of Microbial Virulence Determinants

The picture of the bacterial genome that has emerged over the last several years is of a much more fluid structure than originally envisaged. Evidence from investigations of population structure, phylogeny and large-scale sequencing, has demonstrated that transposition and re-combination events have contributed as much or more than point mutation to the evolution of some bacterial species. Mobile elements such as transposons and bacteriophages have long been known to import novel genes and cause gene disruptions and re-arrangements. More recently, pathogenicity islands have been described in many species. Pathogenicity islands consist of clusters of genes implicated in the virulence of an organism. For example, they frequently harbour the coding sequences for the TTSS described previously. They are believed to arise by horizontal gene transfer on the basis of an altered base composition (in comparison with other genes in the same species), a distribution restricted to virulent strains within a species, and association with transposable elements. Horizontal gene transfer also appears to be responsible for re-arrangements at loci responsible for synthesis of surface antigens including pili, O-antigens and capsules. Again such loci are often characterized by unusual base composition and restricted distribution in the species.

Influx and exchange of genes by transposition and re-combination have had a profound effect on bacterial phenotypes. Incoming DNA provides opportunities for adaptation to novel environments, vividly illustrated by bacterial resistance to antibiotics and the spread of resistance by horizontal gene transfer events. The insertion or re-arrangement of DNA may also have effects on pre-existing chromosomal genes by disrupting open reading frames, altering levels of expression, or shuffling functional protein domains. In the context of survival in a human host, adaptations could include mechanisms to acquire nutrients, to evade or inhibit immune responses, and to colonize new niches. Hence, the set of genes that have a restricted distribution within a bacterial species of clinical importance is likely to include genes that are responsible for differences in pathogenic potential between strains. Recent developments in microarray technology make it possible to undertake a systematic analysis of non-conserved elements in a bacterial genome and their distribution among different strains.

Only a brief examination of the genomes of some of the periodontal organisms currently available suggests that horizontal gene transfer may be a significant force for the evolution of these organisms. For example, the percentage G + C taken at 500 bp intervals along the genomes of *P. gingivalis* W83 (Nelson et al. 2003) and *Treponema denticola* ATCC 35405 (Seshadri et al. 2004) are shown in Fig. 2. It is evident that there are notable differences in the average G + C percentage values at specific points in the genomes of the two organisms, which may indicate arrival of DNA from a ''foreign source'' by one of the mechanisms described above. The contribution of these regions to the pathogenic potential or virulence of each organism is not known although one of these regions in *P. gingivalis* W83, surrounding position 220 kb, contains the coding sequences for a major outer membrane protein, RagB (PG0186), which we have previously shown to be a significant target of the immune response in periodontal patients compared with controls. Furthermore, at least in the case of *P. gingivalis*, it is becoming clear that these regions of the bacterial chromosome may be significantly polymorphic between different strains on the basis of genomic typing by microarray analysis (Curtis et al. unpublished, Chen et al. 2004) and therefore may contribute to the observed variations in virulence potential of different isolates in model systems. Hence, the availability of these genomic sequences provides a significant resource for the identification of novel putative determinants of virulence in these organisms and avenues for future research.

While horizontal gene transfer and acquisition of novel determinants of virulence is now an accepted mechanism of pathogen evolution, there is also newly emerging evidence that gene loss may play an equally significant role. These findings stem from the field of comparative genomic analysis of related species. For example, *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica* are three closely related Gram-negative species which colonize the respiratory tract of mammals. *B. bronchiseptica* has a broad host range and causes chronic and often

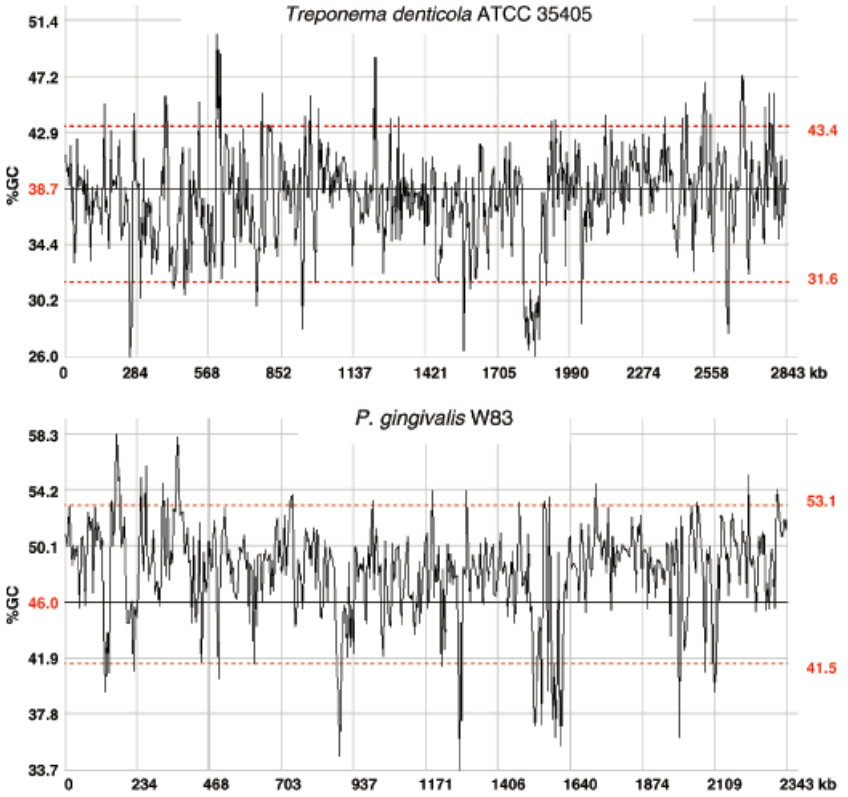

Fig. 2. Percentage G + C plots of the genomes of *Treponema denticola* and *Porphyromonas gingivalis* at 500 bp intervals. Data from www.tigr.org. The average G+C value for *T. denticola* is 38.67 and for *P. gingivalis* is 45.96. The red dashed lines show the 5% and 95% limits of variation.

asymptomatic respiratory infections in a wide range of animals but only occasionally in humans. *B. parapertussis* has a more restricted host range to humans and sheep and in human infants can cause whooping cough. On the other hand, *B. pertussis* is an obligate human pathogen and is the prime aetiologic agent of whooping cough responsible for over a quarter of million deaths annually. Comparative genomic analysis of these three species has demonstrated that *B. pertussis* and *B. parapertussis* are independent ancestors of *B. bronchiseptica* and have experienced significant large-scale gene loss and gene inactivation during their evolution (3816 and 4404 genes in total compared with 5007 genes in *B. bronchiseptica*). Most of the virulence factors, however, are common to all three species. The principal genetic differences which probably account for the increased human pathogenicity of *B. pertussis* are not the presence of novel determinants of virulence, but the loss of regulatory or control functions which may govern the expression of these factors (Parkhill et al. 2003). Similar correlations of gene loss with virulence are now emerging in comparative analysis of other closely related species, for example, the Yersinia (Hinchliffe et al. 2003). Indeed it is possible that the well-established increased levels of leucotoxin by the *A. actinomycetemcomitans* JP2 clone, caused by a deletion in the upstream promoter region of the *ltxA* gene (Brogan et al. 1994) may represent a periodontal example of the link between loss of tight regulatory control of gene expression and increased virulence.

The message, in terms of the critical pathways of microbial virulence in periodontal disease, may be that it is not the particular complement of virulence genes in a periodontal pathogen which dictates its disease potential, but rather the manner in which these genes are regulated and controlled.

Conclusions and Recommendations

There are two main conclusions from this brief review of the microbial pathogenesis of periodontal disease.

To date, the virulence determinants of periodontal organisms have been identified largely by in vitro assessment of the properties of suspected pathogens. Gene products and phenotypic traits associated with the evasion, subversion and manipulation of the host response have received particular attention. Absolute confirmation of the contribution of these properties to the processes of colonization, persistence and disease causation remains elusive. Advances in this area are hindered by the absence of a more accurate model system which properly reflects the chronic and multifaceted infection process. Paradoxically, periodontal disease affects tissues which are among the most accessible for experimental study in the human body. In contrast to most other inflammatory conditions of man, the location of the disease allows longitudinal sampling of both the microbiota and the components of innate and specific immunity arraigned against these organisms, and, importantly, the location is amenable to the use of interventionist approaches to experimentally test the contribution of individual candidate determinants of virulence. The concerted development of this field of study will require the collaboration of oral microbiologists, geneticists and clinical periodontologists in order to fully exploit this potential. However, if realized, the field is well placed to be at the forefront of the study of virulence mechanisms of chronic inflammatory infection processes in humans with a mixed/complex microbial aetiology.

Microbial genomics and utilization of the associated technologies is providing novel avenues for the study of the infectious aetiology of periodontal disease. Lessons from other areas of microbiology are demonstrating the enormous potential of the investment in genome sequencing, genomic typing by microarray and comparative genomic sequence analysis of related species and strains. In oral microbiology, we are still only at the beginning of this process but, with continued investment, the future promises a fuller understanding of the underlying basis of microbial virulence in this disease.

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