Genetic Characteristics and Pathogenic Mechanisms of Periodontal Pathogens

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ABSTRACT

Periodontal disease is caused by a group of bacteria that utilize a variety of strategies and molecular mechanisms to evade or overcome host defenses. Recent research has uncovered new evidence illuminating interesting aspects of the virulence of these bacteria and their genomic variability. This paper summarizes some of the strategies utilized by the major species – *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola*, and *Porphyromonas gingivalis* – implicated in the pathogenesis of periodontal disease. Whole-genome sequencing of 14 diverse *A. actinomycetemcomitans* strains has revealed variations in their genetic content (ranging between 0.4% and 19.5%) and organization. Strikingly, isolates from human periodontal sites showed no genomic changes during persistent colonization. *T. forsythia* manipulates the cytokine responses of macrophages and monocytes through its surface glycosylation. Studies have revealed that bacterial surface-expressed O-linked glycans modulate T-cell responses during periodontal inflammation. Periodontal pathogens belonging to the “red complex” consortium express neuraminidases, which enables them to scavenge sialic acid from host glycoconjugates. Analysis of recent data has demonstrated that the cleaved sialic acid

acts as an important nutrient for bacterial growth and a molecule for the decoration of bacteria surfaces to help evade the host immune attack. In addition, bacterial entry into host cells is also an important prerequisite for the lifestyle of periodontal pathogens such as *P. gingivalis*. Studies have shown that, after its entry into the cell, this bacterium uses multiple sorting pathways destined for autophagy, lysosomes, or recycling pathways. In addition, *P. gingivalis* releases outer membrane vesicles which enter cells *via* endocytosis and cause cellular functional impairment.

INTRODUCTION

Pathogenic bacteria use a variety of strategies and molecular mechanisms to evade host defenses and to enhance their penetration into and colonization of host tissues. Some periodontal pathogens, including *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola*, and *Porphyromonas gingivalis*, have developed sophisticated strategies for host evasion and virulence, such as molecular mimicry of host tissues to avoid immune detection, modulation of the host immune response, and entry into host cells to escape immune surveillance. Recent research has shed further light on some of the properties and mechanisms that contribute to the virulence of these key species. Periodontal bacteria exploit a vast number of mechanisms to interact with human tissues during pathogenesis (Berezow and Darveau, 2011; Hernández *et al.*, 2011; Hajishengallis and Lamont, 2012; Teles *et al.*, 2013). Among them, this paper focuses on four topics.

A. actinomycetemcomitans has long been implicated in periodontitis, but is also found in healthy mouths as part of the commensal flora (Haffajee and Socransky, 1994). Such variations in pathogenicity may be due to genetic variability between and among different strains. Recent studies, outlined in this paper, have investigated this possibility.

Recent studies on an enigmatic and relatively less-studied organism, *T. forsythia*, have revealed that this bacterium induces alveolar bone loss in mice by driving TLR2-dependent polarization of Th cells toward the Th2 subset. This effect depends on the

Key Words

genomic islands, surface glycosylation, neuraminidases, capsule, complement resistance, membrane trafficking.

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Table. *A. actinomycetemcomitans* Strains Included in the Comparative Genomic Analysis

Strain	2nd Strain ^a	Serotype	Race/Ethnicity ^b	Age (yrs)	Gender	Diagnosis ^c	Geographic Location
D7S-1	N.A.	a	Af	29	Female	AP	USA
D17P-3	N.A.	a	As	24	Male	AP	USA
H5P1	N.A.	a	As	27	Female	H	USA
HK1651	N.A.	b/JP2	African	N.A.	N.A.	AP	Ghana
ANH9381	N.A.	b	C	50	Male	H	Finland
I23C	S23A	b	C	48	Male	CP	Finland
SCC1398	SCC4092	b	C	25	Female	AP	Finland
SCC2302	AAS4a	c	C	33	Female	G	Finland
D11S-1	N.A.	c	Af	16	Female	AP	USA
D17P-2	N.A.	c	As	24	Male	AP	USA
I63B	N.A.	d	C	49	N.A.	H	Finland
SCC393	A160	e	C	40	Male	CP	Finland
SC1083	N.A.	e	N.A.	N.A.	N.A.	N.A.	N.A.
D18P-1	N.A.	f	As	20	Female	AP	USA

^aA second strain isolated from the same individual; N.A., not available.

^bAf, African American; As, Asian-American; C, Caucasian.

^cAP, aggressive periodontitis; H, periodontally healthy; CP, chronic periodontitis; G, gingivitis.

bacterium's ability to decorate its surface with unique O-glycan structures that play an immunomodulatory role.

In another kind of surface modification, "red complex" bacteria have been shown to utilize neuraminidases to scavenge host sialic acid for use as a decorating molecule. This tactic helps the pathogens to evade host immune defenses.

Bacterial invasion of host cells has been well documented and is thought to be an important capability for the successful pathogen. The mechanisms by which *P. gingivalis* and its products gain entry into host periodontal cells, their possible intracellular destinations, and the effects of this bacterial occupation are discussed in the following.

COMPARATIVE GENOMICS OF *A. ACTINOMYCETEMCOMITANS*

Gram-negative *A. actinomycetemcomitans* is a facultative bacillus phylogenetically related to a number of mucosal pathogens and commensal bacteria in *Pasteurellaceae*. The organism is considered one of the three or four primary periodontal pathogens and a major cause of localized aggressive periodontitis (Haffajee and Socransky, 1994). However, it is also present as a component of the oral microbiota in periodontally healthy individuals (Slots and Ting, 1999). This implies strain-to-strain variation in their virulence potential. Such variation could be attributed to differences in the gene content and organization in the genome of individual strains (Hacker and Carniel, 2001). It was therefore hypothesized that the *A. actinomycetemcomitans* genome consists of a core gene pool (*i.e.*, genes shared by all strains) and an accessory gene pool (*i.e.*, genes shared by some but not all strains) similar to many other bacterial species (Hacker and Carniel, 2001). The accessory genes, in particular those organized as genomic islands, may encode novel virulence determinants acquired by *A. actinomycetemcomitans* via horizontal gene transfer (HGT). Once identified, the accessory genes and genomic islands may be examined further for their roles in the pathogenesis of periodontitis.

Figure 1. The size of the *A. actinomycetemcomitans* pan genome and the range of the strain-to-strain variation in the gene content of their genomes.

This hypothesis was tested in a series of studies that involved a large set of *A. actinomycetemcomitans* strains (Table) (Kittichotirat *et al.*, 2010, 2011; Sun *et al.*, 2013). First, whole-genome sequencing (WGS) was performed with the 454 platform of 14 clonally, geographically, and clinically diverse strains of *A. actinomycetemcomitans* (Kittichotirat *et al.*, 2011). The sequencing results were subject to gene identification and annotation by standard protocols. The results showed that the pan genome of *A. actinomycetemcomitans* is composed of 3,301 protein-coding genes, which included 2,034 core genes and 1,267 accessory genes (Fig. 1). Approximately three-quarters of the accessory genes were classified as poorly defined by Cluster of Orthologous Groups. The pan genome of *A. actinomycetemcomitans* was open, with 16 new genes expected for each future additional strain sequenced. The variation in gene content between and among strains was 0.4–19.5% of their genomes. In total, 171 genomic islands were identified among the 14 strains and accounted for 61% of accessory genes found among 14 *A. actinomycetemcomitans* strains. The functions of the genomic

islands of *A. actinomycetemcomitans* are largely unknown. However, the utility of comparative genomic analysis for identifying virulence determinants is exemplified in the case of cytolethal distending toxins *cdtABC* (Mayer *et al.*, 1999). Here the analysis clearly identified a large genomic island carrying *cdtABC* in the *A. actinomycetemcomitans* strain D7S-1 but not strain SC1083. Such an insertion/deletion relationship between and among strains defines the boundaries of genomic islands, identifies their gene content, and permits testing of the phenotypes associated with the islands.

In addition to variations in the gene content of the genome, *A. actinomycetemcomitans* strains exhibit remarkable differences in the arrangement of genes in their genomes (Kittichotirat *et al.*, 2010). For example, the difference in the genomic arrangement (calculated as reversal distances) (Tesler, 2002; Darling *et al.*, 2004) between *A. actinomycetemcomitans* serotype a strain D7S-1 and serotype b strain HK1651 was much greater than 14 such intra-species comparisons of 7 randomly chosen bacterial species (Kittichotirat *et al.*, 2010). Such variations in gene position could have an impact on gene expression profiles or bacterial growth, due to the interaction between bacterial cellular processes and chromosomes (Rocha, 2008). With detailed genomic information available, one could design a specific hypothesis to test the relationship between gene position and expression in *A. actinomycetemcomitans*.

Finally, the gains and losses of genes during persistent colonization of *A. actinomycetemcomitans* in human periodontal sites were assessed (Sun *et al.*, 2013). WGS was combined by comparative genomic hybridization with microarray, transcriptomic profiling by microarray, and PCR analysis to examine the genomes of four pairs of *A. actinomycetemcomitans* strains. The two strains in a pair were isolated from the same individual and were confirmed to be clonally identical. The results showed very limited genomic changes. More importantly, none of the genomic islands exhibited gains or losses of genes. Transcriptome profiling showed little or no difference in the paired strains. The relative stability of genomic islands may support the hypothesis that they provide critical functions for the bacteria and therefore become fixed in the genome after horizontal gene transfer.

T. forsythia SURFACE GLYCOSYLATION: A STRATEGY FOR MANIPULATING HOST IMMUNITY

T. forsythia – a Significantly Overlooked Culprit

T. forsythia is another species that has been implicated as an etiologic agent of periodontitis. This species is often found cohabiting with *P. gingivalis* and *T. denticola* in subgingival dental plaque biofilms (Tanner and Izard, 2006). Recent studies have also detected *T. forsythia* in acute and chronic endodontic infections (Saito *et al.*, 2009; Montagner *et al.*, 2012). Increased

Figure 2. (A) TEM micrographs of *T. forsythia* ultrathin sections (courtesy of Dr. G. Stafford, University of Sheffield, UK). (B) O-glycan core structure linked to a peptide motif in surface proteins. WecC is involved in the synthesis of a tri-saccharide branch (circled) on the O-glycan core (adapted from Posch *et al.* 2011).

numbers of *T. forsythia* are reported in subgingival plaque samples from post-menopausal obese women (Brennan *et al.*, 2007; Haffajee and Socransky, 2009) and patients with chronic periodontitis and type 2 diabetes (Li *et al.*, 2013). *T. forsythia* is also linked to systemic disease such as cardiovascular diseases (Figuerio *et al.*, 2011; Leishman *et al.*, 2012) and arthritis (Moen *et al.*, 2006; Scher *et al.*, 2012). Interestingly, domestic cats could become carriers (reservoirs) of *T. forsythia* and transmit the bacterium to their owners (Booij-Vrieling *et al.*, 2010).

T. forsythia has remained an under-investigated organism because of its fastidious growth and recalcitrant nature to genetic manipulation. In spite of these difficulties, several investigations on the role of this species in the etiology of periodontitis have been conducted (Sharma, 2010, 2011; Settem *et al.*, 2012a; Stafford *et al.*, 2012).

Tannerella's Sugary Coat

Pathogens maintain their survival in the host through manipulation of the host immune defenses. Toward that end, microbes sometimes decorate their surfaces with unique polysaccharides (sugars), which modulate host-microbe interactions at the immune interface. *T. forsythia* possesses a surface (S)-layer (Tanner *et al.*, 1986), visible as a striated "tooth-like" coat on the bacterium's surface, with an approximate thickness of 25 nm (Fig. 2A). It is composed of 2 high-molecular-weight proteins, TfsA (200 kDa) and TfsB (210 kDa), abundantly glycosylated with N- and O-linked sugars (Lee *et al.*, 2006). A genetic operon involved in the glycosylation of these proteins has been identified (Honma *et al.*, 2007), and the *wecC* gene encoding a homolog of UDP-N-acetylmannosaminuronic acid dehydrogenase associated with this operon was later shown to be involved in the terminal modification of a protein-linked O-glycan core with a tri-saccharide motif consisting of two sub-terminal mannosuronic acid residues and a terminal pseudaminic acid residue (Posch *et al.*, 2011) (Fig. 2B).

Manipulating Immunity

In vitro studies utilizing mutants lacking the S-layer suggested that the S-layer helps to delay the ability of macrophages and monocytes to mount a cytokine response (Sekot *et al.*, 2011). Subsequent studies showed that the terminal tri-saccharide motif (two sub-terminal mannosuronic acid residues and a terminal pseudaminic acid residue; Fig. 2B) on the O-glycan core linked to surface proteins in *Tannerella* affects the ability of the bacterium to regulate cytokine secretion in immune cells (Settem *et al.*, 2012b). The ED1 strain lacking this O-glycan motif induced significantly higher amounts of IL-6 and IL-1 β secretion in mouse bone-marrow-derived dendritic cells (mBMDCs) and macrophages compared with wild-type *T. forsythia* cells. Interestingly, IL-23 (Th17-activating cytokine) levels increased in both mouse macrophages and mBMDCs challenged with the ED1 strain compared with the wild-type strain. In addition, the loss of the O-glycan motif resulted in increased processing of bacteria by DCs (Settem *et al.*, 2012b).

To ascertain if the surface O-glycans influence the bacterium's pathogenicity *in vivo*, investigators infected BALB/c mice with the wild-type or ED1 mutant cells by oral gavage and evaluated the bacterial colonization, tissue inflammation, and alveolar bone loss parameters post-infection. Mice infected with the wild-type *T. forsythia* strain showed higher alveolar bone loss than the ED1-infected mice (Settem *et al.*, 2012b). In addition, the osteoclastic activity correlated with the alveolar bone loss; significantly fewer tartrate-resistant acid phosphatase (TRAP)-positive cells were observed in jawbone sections of mice infected with the medium (sham) or ED1 strain, compared with the wild-type strain. In line with the increased ability of ED1 to induce inflammatory cytokines in monocytes/macrophages, this mutant also caused increased infiltration of CD45⁺ lymphocytes in gingival tissues. Moreover, infection with the wild-type strain skewed the CD4⁺ T-cell differentiation toward Th2, whereas the ED1 infection resulted in skewing toward the Th17 subset. Furthermore, Th17 polarization in the ED1-infected mice paralleled increased neutrophil infiltration into the mouse gingival tissues. A consequence of the increased neutrophil infiltration was decreased persistence of the ED1 strain compared with that in the wild-type strain, judged by quantitative PCR for *T. forsythia* DNA in gingival tissues post-infection. Strikingly, under these conditions, the Th17 response was not associated with enhanced alveolar bone osteoclastic activity, in contrast to the role of Th17 in a rheumatoid arthritis setting (Onishi and Gaffen, 2010).

NEURAMINIDASE — A NEW PLAYER IN THE PATHOGENESIS OF PERIODONTITIS

Sialic acid is a group of structurally related nine-carbon sugar acids that feature prominently at terminal positions of many eukaryotic surface-exposed glycoconjugates, where they are involved in a wide range of biological processes, including cell-cell interactions and small-molecule-cell recognition (Vimr *et al.*, 2004). Several bacterial pathogens have evolved to utilize sialic acid as decorating molecules to modify their surface-exposed macromolecules, such as lipopolysaccharides (LPS) and polysialic acid (PSA) capsules. Such modifications allow bacterial pathogens to disguise themselves and thus circumvent

and/or counteract the host's immune responses (Severi *et al.*, 2007). Bacteria acquire sialic acid either *via a de novo* biosynthesis pathway or by means of a scavenger pathway that is primarily mediated by neuraminidases. Neuraminidase (also referred to as sialidase) is a family of enzymes that catalyzes the removal of terminal sialic acid from glycoconjugates (Vimr and Lichtensteiger, 2002). Various mucosal pathogens, ranging from *Streptococcus pneumoniae* in the airway to *Vibrio cholerae* in the gut, utilize neuraminidases to scavenge host sialic acid. In these bacteria, neuraminidases are often associated with their virulence (Galen *et al.*, 1992; Uchiyama *et al.*, 2009). Neuraminidase activity is also observed in several oral bacteria, including the "red complex" bacteria (Moncla and Braham, 1989). In addition, Gibbons *et al.* reported that neuraminidase treatment could expose cryptic receptor which in turn increases the attachment of *P. gingivalis* and other oral pathogens to gingival epithelial cells (Gibbons *et al.*, 1990).

Neuraminidase Activity in the "Red Complex" Bacteria

Similar to other mucosal pathogens, periodontal pathogens such as the "red complex" bacteria possess neuraminidase activity. For instance, *T. forsythia* encodes two neuraminidases (NanH and SiaH). NanH (TF0035) is a major neuraminidase, and its deletion mutant fails to attach to and invade human gingival epithelial cells (Honma *et al.*, 2011). *P. gingivalis* encodes at least one homologue (PG0352) of neuraminidase. Recent studies showed that PG0352 is involved in biofilm formation, capsule synthesis (Fig. 3, left panel), serum resistance, and the pathogenicity of *P. gingivalis* (Aruni *et al.*, 2011; Li *et al.*, 2012). Similar to *P. gingivalis*, *T. denticola* also encodes a putative neuraminidase (TDE0471). Experimental evidence has recently shown that TDE0471 is a neuraminidase that removes sialic acid from human serum glycoconjugates, and that it affects nutrient acquisition, complement activation (Fig. 3, right panel), and the virulence of *T. denticola* (Kurniyati *et al.*, 2013).

As periodontal pathogens, the "red complex" bacteria primarily inhabit the gingival crevices bathed in plasma, which contains a high concentration of sialic acid that is bound to a diverse range of glycans. In this unique niche, utilization of host sialic acid by a bacterium would confer on it a survival advantage. In this regard, it is not surprising that all 3 "red complex" bacteria possess neuraminidases. Recent studies have demonstrated that the "red complex" bacteria use neuraminidases to scavenge host sialic acid, which can benefit the pathogens in at least two ways: nutrient acquisition and immune evasion (Aruni *et al.*, 2011; Honma *et al.*, 2011; Li *et al.*, 2012; Kurniyati *et al.*, 2013). Interestingly, these neuraminidases are surfaced-exposed and share similar biochemical features. Thus, the neuraminidases may be an ideal target for the development of new therapeutic agents for the intervention and prevention of periodontitis, *e.g.*, the design of specific inhibitors against neuraminidases.

Potential Effects of Neuraminidases on Innate and Adaptive Immunity

Interestingly, the surfaces of host cells involved in immune systems contain a family of sialic-acid-binding immunoglobulin-like lectins (Siglecs), which play critical roles in regulating the functions of the

Figure 3. Neuraminidases affect capsule synthesis of *P. gingivalis* (left panel) and complement activation and deposition of *T. denticola* (right panel). (For details, see Li *et al.*, 2012, and Kurniyati *et al.*, 2013.) (**left panel**) One central slice of a tomographic reconstruction from Pg83 (wild type) and ΔPG352 (a neuraminidase-deficient mutant) is shown in panels (A) and (B), respectively. (C, D) Corresponding surface views of the reconstructions from Pg83 and ΔPG352, respectively. (E) The proteinase K treatment (200 μg/mL for 40 min at 37°C) does not remove the thin CPS layer of the ΔPG352 mutant. (F) Supplementing with Neu5Ac (30 μg/mL) restored the CPS formation in the ΔPG352 mutant. The thickness of the CPS layer in the mutant is about 30 nm, which is similar to that of Pg83 (G). The numbers represent the thickness of CPS. (**right panel**) Deposition of membrane attack complex (MAC) on *T. denticola* strains: Td35405 (wild type), Tde471^{mut} (a neuraminidase-deficient mutant), and Tde471^{com} (a cis-complemented strain). A 10⁶ quantity of bacterial cells was co-incubated with 25% normal human serum (NHS) for 20 min at 37°C. As a control, 10 mM EDTA was added to the reactions to block complement activation. The resultant serum-treated cells were subjected to SDS-PAGE, followed by Coomassie blue staining (A), or probed with 3 different antibodies as labeled: (B) αC3, a polyclonal antibody against C3; (C) αC9, a monoclonal antibody against C9; and (D) αC5-9, a monoclonal antibody against the C5-9 complex. Notably, complement components were detected only in the serum-treated Tde471^{mut} but not in the serum-treated Td35405 and Tde471^{com} strains. The complement factors detected by the antibodies and their molecular weights are indicated.

cells in innate and adaptive immune systems *via* glycan recognition (Crocker *et al.*, 2007). It is intriguing to consider the potential effects that neuraminidases could impose on innate and adaptive immunity regulated by the Siglecs. Furthermore, sialylation is essential for the anti-inflammatory activity of human plasma immunoglobulin G (also referred to as intravenous IgG, IVIG), which is often utilized to treat a variety of human hematological and immunological disorders. Recent studies have shown that desialylation of IVIG not only depletes its anti-inflammatory activity but also potentially leads to the generation of pathogenic antibodies (Kaneko *et al.*, 2006). It is even more intriguing to consider the potential impact of neuraminidases on IgG in the GCF and their role in the pathogenesis of periodontitis, as well as in the systemic diseases associated with periodontitis.

CELLULAR MEMBRANE TRAFFICKING OF INTRACELLULAR *P. gingivalis*

Intracellular Infection

Bacterial entry into host cells allows pathogens to occupy various niches within the human body, a process required for

successful establishment of bacterial infection. Confocal scanning laser microscopy (CSLM) showed the intracellular localization of several periodontal bacteria (Lamont and Yilmaz, 2002), while a method that used *in situ* hybridization with 16S rRNA gene probes and CSLM detected *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythia*, and *T. denticola* within epithelial cells obtained from periodontal pockets, gingival crevices, and buccal mucosa collected from individuals with or without chronic marginal periodontitis (Colombo *et al.*, 2007). Thus, most periodontal pathogens likely have an ability to enter periodontal cells.

Intracellular Dynamics of *P. gingivalis*

P. gingivalis can enter host cells, including epithelial and endothelial cells (Amano *et al.*, 2010a). *P. gingivalis* fimbriae specifically interact with α5β1-integrin of epithelial cells. The bacterium is then captured by cellular pseudopodia, which enables invagination to occur through the endosomal pathway. This entry event requires host cellular dynamin, actin fibers, microtubules, and lipid rafts. Following entry into the cells, it has not been clearly demonstrated how *P. gingivalis* trafficks within the infected cells. Intracellular

Figure 4. Proposed model of *P. gingivalis* trafficking in immortalized human epithelial cells. *P. gingivalis* initially localized within an endocytic vacuole (early endosome) after entry. Some bacteria are routed to late endosomes, then subsequently sorted to lysosomes for degradation. Other bacteria likely promote their own entry into the autophagic pathway by bacterial escape from endosomes or fusion of endosomes with autophagosomes, and then are sorted to autolysosomes, which are formed by the fusion of autophagosomes with lysosomes. Some intracellular *P. gingivalis* organisms seem able to escape from endosomes via the recycling pathway for exocytosis. Subsequently, the bacteria exit from primarily infected host cells into the intercellular space and enter new host cells, which facilitates further penetration of the host tissue in a transcellular manner.

P. gingivalis is reportedly localized in various cellular compartments, such as the cytoplasm, endosomes, and autophagosomes, with autophagosome localization reported to occur in endothelial and smooth-muscle cells, but not in gingival epithelial cells (reviewed by Amano *et al.*, 2010a). In addition, the bacterium has not been found in the cytoplasmic spaces of endothelial cells. Thus, the intracellular localization of *P. gingivalis* might be specific to the type of host cell.

We showed that *P. gingivalis* was internalized with early endosomes positive for the FYVE domain of EEA1 (an early endosome marker) and transferrin receptor, and about half of the intracellular bacteria are sorted to lytic compartments, including autolysosomes and late endosomes/lysosomes, in immortalized human gingival epithelial cells (Amano and Furuta, 2012) (Fig. 4). Meanwhile, a considerable number of the remaining organisms are sorted to Rab11- and RalA-positive recycling endosomes, which mediate the bacterial exit from infected cells (Takeuchi *et al.*, 2011). Inhibition experiments revealed that bacterial exit is dependent on actin polymerization, lipid rafts, and microtubule assembly. Furthermore, dominant-negative forms and RNAi-knockdown of Rab11, RalA, and exocyst complex subunits (Sec5, Sec6, and Exo84) significantly disturbed the exit of *P. gingivalis*. It should be noted that intracellular bacteria were sorted to three different destinations within the same cells and that the bacterial exit likely allowed for the further penetration of periodontal tissues. These results imply the existence of unknown mechanism(s) that alternate multiple sorting pathways destined for autophagy, lysosomes, or the recycling pathway.

Figure 5. Outer membrane vesicles (MVVs) secreted from *P. gingivalis* entering human epithelial cells. Human epithelial cells were incubated with *P. gingivalis* strain TDC60 for 15 min, and bacterial entry was observed by transmission electron microscopy. Arrowheads indicate MVVs on the surface of a *P. gingivalis* organism of which entry was triggered by cellular membrane ruffling.

Cellular Impairment by Intracellular *P. gingivalis*

Cellular integrins mediate specific attachment between a cell and the extracellular matrix, activate the intracellular cytoskeleton network, and elicit various intracellular events, *i.e.*, cellular anchorage, directed migration, wound healing, regeneration, and tissue integrity (Hakkinen *et al.*, 2000). During these processes, the outside-in signaling of integrins, paxillin, and focal adhesion kinase (FAK) play important roles. Intracellular *P. gingivalis* has been shown to degrade paxillin and FAK, resulting in impaired cellular function during wound-healing and periodontal tissue regeneration (Kato *et al.*, 2007).

Entry Mechanism of Outer Membrane Vesicles of *P. gingivalis*

A great many Gram-negative bacteria, including *P. gingivalis*, have an ability to release outer membrane vesicles (MVVs) in an extracellular manner, as noted above (Amano *et al.*, 2010b) (Fig. 5). *P. gingivalis* MVVs retain major components of outer membrane constituents, including LPS, muramic acid, capsule, fimbriae, and proteases termed “gingipains.” Fimbriae mediate bacterial adherence to and entry into host cells (Enersen *et al.*, 2013), while gingipains contribute to the destruction of periodontal tissues (Kadowaki *et al.*, 2007). *P. gingivalis* MVVs swiftly entered gingival epithelial cells *via* lipid rafts through the actin filament assembly, which is dependent on phosphatidylinositol 3-kinase and Rac1, whereas the clathrin- and dynamin-dependent pathways were not involved in MV entry. Next, the MVVs were routed to early endosomes and subsequently sorted within 90 min to lysosomes, where they remained for more than 24 hr (Furuta *et al.*, 2009b). Although taken up by the cellular digestive machinery, MVVs degraded the cellular

transferrin receptor and focal adhesion complex proteins, which resulted in depletion of intracellular transferrin and inhibition of cellular migration (Furuta *et al.*, 2009a). These findings suggest that *P. gingivalis* MVs enter host cells via an endocytosis pathway and that MV-associated gingipains degrade cellular functional molecules, resulting in cellular impairment. This indicates a novel role for *P. gingivalis* MVs in the etiology of periodontitis.

CONCLUSION

Ongoing research is increasing our understanding of the factors that contribute to the pathogenic behavior of some periodontal bacteria, including their genetic characteristics and their interactions with the host environment.

A. actinomycetemcomitans strains are remarkably diverse in gene content and gene organization of their genomes. This diversity can be used to design hypothesis-driven experiments to understand the molecular basis for intra-species variations in the virulence potential of *A. actinomycetemcomitans*. The results may also serve as an example for the evolution and adaptation of oral bacteria to humans.

Studies have demonstrated that *T. forsythia* induces alveolar bone loss in mice by driving the TLR2-Th2 inflammatory axis. The pathogenicity of the organism is dependent on its ability to decorate the surface with a unique glycan motif, which plays a vital role in modulating the cytokine responses of antigen-presenting cells to ensure subsequent blockade of the Th17 and induction of Th2 responses. An understanding of how *T. forsythia* shapes the immunity conducive for its survival, and possibly that of cohabiting pathogens, will provide better insight into the pathobiology of periodontitis and facilitate the future development of glycan-based immunotherapies in the prevention of periodontitis.

Accumulating evidence shows that neuraminidases play critical roles in the physiology and pathogenicity of “red complex” bacteria and may be an important new player in the development and progression of periodontitis. Further research is required to investigate the feasibility and therapeutic potential of agents targeting bacterial neuraminidases.

Analyses of bacterial strategies used by *P. gingivalis* to determine intercellular persistence, dissemination, and fate are well-advanced, though we still know only a part of the picture. Efficient bacterial entry into host cells is considered to be tightly related to cytotoxicity expressed by periodontal pathogens. However, recent studies showed that human oral epithelial cells harbor large masses of intracellular bacterial consortia, resembling the polymicrobial nature of tooth-surface biofilm (Colombo *et al.*, 2007). In addition, no significant induction of apoptosis or necrosis was observed in epithelial cells heavily invaded by bacteria (Rudney *et al.*, 2005). These new findings may represent a periodontal bacterial intracellular lifestyle characterized by rest and quiet, which allows them to persist in, but not destroy, the host.

These sophisticated strategies of the pathogens contribute to periodontal destruction, which could be further enhanced by the bacterial mutual communication with a complex polymicrobial etiology (Hajishengallis and Lamont, 2012). We must move ahead powerfully toward an in-depth understanding of periodontal pathogenesis and new targets for therapeutic intervention.

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