Published in final edited form as:
Mol Oral Microbiol. 2010 October ; 25(5): 317–330. doi:10.1111/j.2041-1014.2010.00583.x.

Tannerella forsythia infection-induced calvarial bone and soft tissue transcriptional profiles

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SUMMARY

Tannerella forsythia is associated with subgingival biofilms in adult periodontitis, although the molecular mechanisms contributing to chronic inflammation and loss of periodontal bone remain unclear. We examined changes in the host transcriptional profiles during a *T. forsythia* infection using a murine calvarial model of inflammation and bone resorption. *Tannerella forsythia* was injected into the subcutaneous soft tissue over calvariae of BALB/c mice for 3 days, after which, the soft tissues and calvarial bones were excised. RNA was isolated and Murine GeneChip® array analysis of transcript profiles showed that 3226 genes were differentially expressed in the infected soft tissues ($P < 0.05$) and 2586 genes were differentially transcribed in calvarial bones after infection. Quantitative real-time reverse transcription-polymerase chain reaction analysis of transcription levels of selected genes corresponded well with the microarray results. Biological pathways significantly impacted by *T. forsythia* infection in calvarial bone and soft tissue included leukocyte transendothelial migration, cell adhesion molecules (immune system), extracellular matrix–receptor interaction, adherens junction, and antigen processing and presentation. Histologic examination revealed intense inflammation and increased osteoclasts in calvarias compared with controls. In conclusion, localized *T. forsythia* infection differentially induces

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Disclosures
The authors have no financial conflict of interest.

SUPPORTING INFORMATION
Table S1. Leave-one-out cross-validation – calvarial bone.
Table S2. Leave-one-out cross-validation – calvarial soft tissue.
Table S3. Comparison of expression of selected genes in calvarial tissue and calvariae (high dose) by microarray and real-time quantitative polymerase chain reaction.
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transcription of a broad array of host genes, and the profiles differ between inflamed soft tissues and calvarial bone.

Keywords

calvarial bone; gene expression; microarray; *Tannerella forsythia*; tissue

INTRODUCTION

Periodontitis is a chronic immunoinflammatory infectious disease initiated by complex microbial subgingival biofilms resulting in destruction of periodontal tissues and resorption of alveolar bone. *Tannerella forsythia*, an oral, gram-negative, filament-shaped, non-pigmented, strict anaerobe, is an important member of pathogenic biofilms at sites of periodontal disease (Socransky *et al.*, 1998; Tanner *et al.*, 1998; Tanner & Izard, 2006). A strong association with chronic periodontitis has been shown for *T. forsythia* and *Porphyromonas gingivalis* (Haffajee *et al.*, 2006). Several potential virulence factors of *T. forsythia* have been reported, including PrtH cysteine protease (Saito *et al.*, 1997), forsythia-detaching factor (Nakajima *et al.*, 2006), a sialidase (Ishikura *et al.*, 2003), an apoptosis-inducing activity (Arakawa *et al.*, 2000), a hemagglutinin (Murakami *et al.*, 2002), alpha-D-glucosidase and *N*-acetyl-β-glucosaminidase (Hughes *et al.*, 2003), production of methylglyoxal (Maiden *et al.*, 2004), matrix metalloproteinase (MMP) -like enzyme karilysin (Karim *et al.*, 2010), S-layer mediating hemagglutination, adhesion/invasion of epithelial cells, and murine subcutaneous abscess formation (Sabet *et al.*, 2003). *Tannerella forsythia* also expresses a cell surface-associated and secreted protein BspA (Sharma *et al.*, 1998), which has been recognized as an important virulence factor in inducing alveolar bone loss in mice (Sharma *et al.*, 2005). Furthermore, BspA is an important modulator of host innate immune responses through activation of Toll-like receptor 2 in human gingival epithelial cells in cooperation with Toll-like receptor 1 (Onishi *et al.*, 2008). In addition, twelve *in vivo*-induced genes (antigens) of *T. forsythia* were determined from sera of patients with periodontal disease using *in vivo*-induced antigen technology (Yoo *et al.*, 2007). Recent reports indicate synergy between *T. forsythia* and *Fusobacterium nucleatum* in biofilm formation (Sharma *et al.*, 2005). Moreover, *P. gingivalis* or its outer membrane vesicles enhance the attachment and invasion of *T. forsythia* to human oral epithelial cells (Inagaki *et al.*, 2006), and growth of *P. gingivalis* is stimulated by cell extracts from *T. forsythia* (Yoneda *et al.*, 2005). However, robust data on the *in vivo* role of potential virulence factors, as well as the broader aspects of the host response to virulence factors of *T. forsythia* in the periodontium remain to be defined.

The use of complementary DNA microarrays to survey transcriptional host responses after exposure to microbial pathogens has become a powerful approach to enhance understanding of the molecular basis of the host response to bacterial infections, which is critical for limiting tissue destruction. Host response profiling has identified transcripts uniquely affected by pathogens such as *Listeria monocytogenes*, *Brucella abortus*, and *Mycobacterium tuberculosis* (Cohen *et al.*, 2000, Eskra *et al.*, 2003, Rachman *et al.*, 2006). Recent microarray studies have determined *in vitro* responses of host cells to challenge with *P. gingivalis* or its virulence components in primary human coronary artery endothelial cells and human aortic endothelial cells (Chou *et al.*, 2005), gingival fibroblasts from healthy and inflammatory gingival tissues (Wang *et al.*, 2003), gingival transcriptome patterns during induction and resolution of experimental gingivitis in humans (Offenbacher *et al.*, 2009), and human gingival epithelial cells (Handfield *et al.*, 2005). A recent gingival transcriptome study suggested that the microbial content of the periodontal pocket is a determinant of gene expression in the gingival tissues and provides new insights into the differential ability of

periodontal species to elicit a local host response (Papapanou *et al.*, 2009). In addition, gene ontology analysis of healthy and diseased gingival tissues from patients with advanced periodontitis identified 61 differentially expressed groups including apoptosis, antimicrobial immune response, and antigen presentation (Demmer *et al.*, 2008). A recent review highlighted organism-specific transcriptional responses of gingival epithelial cell responses that correlated with the pathogenic potential of the real bacteria (Handfield *et al.*, 2005). Nevertheless, there remains no report documenting *T. forsythia* induction of gene expression when the microorganism interacts with animal host cells *in vivo*. The aim of the present study was to define changes in the transcriptome of calvarial bone and overlying soft tissues in response to localized *T. forsythia* infection in mice using an established calvarial model of inflammation and bone resorption. We performed a genome-wide transcriptional analysis of the calvarial bone and overlying soft tissues isolated from *T. forsythia*-infected and sham-infected mice. Microarray data analysis identified several hundred altered probe sets, leading to subsequent identification of numerous pathways which were significantly changed in *T. forsythia*-infected mice.

METHODS

Mice

BALB/c female mice 8–10 weeks old (Harlan, Indianapolis, IN) were routinely acclimatized for at least 1 week before use. Mice were infected with *T. forsythia* ATCC 43037 cells as described below following isoflurane inhalation anesthesia. All mouse infection procedures were performed in accordance with the approved guidelines set forth by the Institutional Animal Care and Use Committee at the University of Kentucky (Lexington, KY).

Microorganism and mouse infection

Tannerella forsythia ATCC 43037 cells were grown in trypticase soy agar II basal media supplemented with yeast extract, phytone peptone, sheep blood (5%), and *N*-acetylmuramic acid for 3 days. Bacteria were scraped from the agar surface and resuspended in reduced transport fluid as described previously (Sharma *et al.*, 2005). *Tannerella forsythia* were injected at 1.5×10^9 ($n = 10$ mice) into the soft tissues overlying the calvariae of the mice (Zubery *et al.*, 1998). Bacterial infection, mouse euthanasia, collection, and preparation of calvarial bone and soft tissue for histology were performed as previously described (Zubery *et al.*, 1998; Meka *et al.*, 2010; Bakthavatchalu *et al.*, 2010).

RNA isolation and mouse GeneChip hybridization

RNA isolation, assessment of quality of the RNA preparation, complementary RNA transcription, hybridization to the mouse GeneChip MG-MOE430A (Affymetrix, Santa Clara, CA), and scanning using an Affymetrix GCS 3000 7G Scanner were performed as previously described ((Meka *et al.*, 2010; Bakthavatchalu *et al.*, 2010).

Microarray data analysis

The *T. forsythia* microarray data were normalized, the dataset was evaluated by both unsupervised and supervised analyses, and hierarchal clustering analysis was performed, following which differences between the various treatment tissue classes, and determination of fold-change of significantly impacted genes were determined as previously described (Eisen *et al.*, 1998; Li & Wong, 2001; Feezor *et al.*, 2003; Handfield *et al.*, 2005, 2008; Draghici *et al.*, 2007, Khatri *et al.*, 2007; Hasegawa *et al.*, 2008; Bakthavatchalu *et al.*, 2010; Meka *et al.*, 2010).

Quantitative real-time reverse transcription-polymerase chain reaction analysis

Differential expression of selected genes, that showed significant variation in the microarray analyses, was confirmed by quantitative reverse trasncription–polymerase chain reaction (qRT-PCR) analysis as previously described (Piana *et al.*, 2008; Bakthavatchalu *et al.*, 2010; Meka *et al.*, 2010). Seven representative upregulated genes based on a broad overview of the different functional category such as extracellular matrix, cell adhesion, cell proliferation, immune and defense responses, transport, and other category functions from both soft tissue and calvarial bone were evaluated. These genes were: pleiotrophin (*Ptn*), tumor necrosis factor receptor superfamily 19 (*Tnfrsf19*), periostin (*Postn*) from calvarial tissue and schafen (*Slfn3*), procollagen type I α1 (*Col1a1*), fibronectin (*Fn*), and matrix metalloproteinases 13 (*Mmp13*) from calvarial bone.

Calvarial bone histology

The mouse calvariae were fixed in 10% phosphate-buffered formalin, decalcified, embedded, sectioned, stained, and analysed for osteoclasts as previously described (Zubery *et al.*, 1998; Bakthavatchalu *et al.*, 2010; Meka *et al.*, 2010).

Statistical analyses

Microarray data were analysed as described above. *P*-values of 0.05 or less were considered significant. The qRT-PCR data from two separate experiments were combined and results were expressed as means ± SD.

Microarray data accession numbers

The array results have been deposited in the GEO repository (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession numbers GSE17110 and GSE20389.

RESULTS

Ontology of gene expression changes in murine calvarial soft tissue and bone

The mouse gene chip MOE430A contains 22,690 probe sets, with 17,395 and 17,274 probe sets ($P < 0.001$) providing positive readable signals to *T. forsythia* infection in calvarial soft tissue and bone, respectively. Significant differences were observed in mean gene expression levels of 3226 and 2586 probe sets in soft tissue and bone in response to infections ($P < 0.05$), respectively. Of the significantly regulated genes, 1528 were upregulated and 1698 were downregulated for soft tissue. In calvarial bone samples 1780 genes were upregulated and 806 were downregulated. The results of this initial gene profile analysis demonstrate that *T. forsythia* stimulated greater changes in the transcriptome of upregulated and downregulated genes in soft tissue compared with calvarial bone. The majority of genes with altered expression in calvarial soft tissue to *T. forsythia* infection were primarily associated with basic cellular functions [transcription, cell proliferation, cell cycle, transport, cell adhesion, extracellular matrix (ECM), apoptosis] for maintaining tissue integrity.

The ability of probe sets significant at $P \leq 0.05$ to correctly identify differences between treatment groups was confirmed by leave-one-out cross-validation analysis (see Supporting Information, Tables S1 and S2). The significantly regulated probe sets were analysed by the PATHWAY EXPRESS tool as previously described (Draghici *et al.*, 2007; Khatri *et al.*, 2007). Pathways significantly impacted by *T. forsythia* at the $P \leq 0.05$ level in bone and soft tissue types included: leukocyte transendothelial migration (LTM) (actin cytoskeleton, leukocyte) (Figs. 1 and 2), cell adhesion molecules (CAM) (immune system comprising

antigen-presenting cells, T cells, and B cells) (Fig. 3), ECM–receptor interaction (ECM, integrin, VLA proteins, leukoproteins, cytoadhesin, focal adhesion, proteoglycan, glycoprotein) (Fig. 4), and focal adhesion (ECM–receptor, cytokine–cytokine receptor, actin cytoskeleton, cell proliferation, cell cycle, and three signaling pathway system) (Fig. 5). Table 1 shows soft tissue and calvarial bone pathways generated from this analysis that were predominantly affected in order of their impact factors. *Tannerella forsythia* significantly impacted 14 pathways in calvarial bone and 15 in soft tissue with an impact factor more than five. The high impact factors associated with these pathways predict that the effects of *T. forsythia*-induced gene expression changes in the bone or tissue will have a significant biological effect downstream. There are more than nine pathways, including LTM, CAM, focal adhesion, adherens junction, antigen processing and presentation, and phosphatidylinositol signaling system, that were significantly impacted or overlapped in both the calvarial bone and soft tissue (Table 1), and hence these were chosen for further study to compare and contrast the impact of *T. forsythia* on two distinct tissue types.

Validation of microarray gene expression

Changes in *T. forsythia*-induced transcript expression levels of selected genes from the microarray studies were confirmed by qRT-PCR, including *Ptn*, *Tnfrsf19*, and *Postn* in calvarial tissue and *Slfn3*, *Col1a1*, *Fn*, and *Mmp13* in calvarial bone using aliquots of the pooled RNA samples that were evaluated in the microarrays (see Supporting Information, Table S3). Transcripts of β-actin were used as an expression control and the qRT-PCR analyses were performed at least twice for each gene. The qRT-PCR results confirmed the microarray data and the selected upregulated genes in microarray showed corresponding increased expressions with qRT-PCR analysis, although some expression levels differed between the two techniques.

Inflammatory and immune response gene expression profiles

Since several studies of infection with oral pathogens have emphasized the chronic inflammatory nature of the challenge leading to localized tissue destruction, we also focused on examination of gene profiles related to inflammatory and immune responses. The proinflammatory cytokines, interleukin-1β (IL-1β), IL-6, tumor necrosis factor, and other cytokines such as IL-7, IL-15, IL-17, IL-18, IL-23 involved in induction of inflammation and calvarial bone resorption were not significantly altered in soft tissue and calvarial bone following *T. forsythia* infection. The majority of genes upregulated in calvarial bone during infection were associated with ECM, cell adhesion, cell proliferation, immune and defense responses, transport, and other category functions. The upregulated immune and defense response genes included multiple genes associated with the adaptive immune response regulation of humoral immunity, e.g. *Igh-6*, *Igh*, *Igk-C*, *Igk-V8*, *Igj*, *Igh-VJ558*, and *Camp*. Expression of the extracellular matrix protein gene chondroadherin (*Chad*), matrix metalloproteinases (*Mmp3*, *Mmp13*, *Mmp9*, *Mmp14)*, several types of procollagen (*Colla1*), matrilin (*Matn2*), tissue inhibitor of metalloproteinase (*Timp1*, *Timp2*), various growth factors (*Ctgf*, *Ltbp1*), and especially periostin (*Postn*; three-fold) were upregulated. Increased expression of matrix metalloproteinases 13 (five-fold increase), 9 (11-fold increase), 8 (two-fold increase), 2 (two-fold increase), and 14 (two-fold increase), (*Mmp13*, *Mmp8*, *Mmp2*, *Mmp9*, *Mmp14*) and of cathepsins K (seven-fold increase), G (12-fold increase), E (three-fold increase), C (two-fold increase), H (two-fold increase), and Z (two-fold increase) genes showed upregulation in calvarial bone. In contrast, expression of several *Mmp* genes, including *Mmp3* (0.3-fold decrease), *Mmp8* (0.2-fold decrease), *Mmp9* (0.2-fold decrease), *Mmp10* (0.5-fold decrease), *Mmp13* (0.2-fold decrease), *Mmp14* (0.4-fold decrease), *Mmp19* (0.3-fold decrease) and cathepsin genes, *Ctsl* (0.5 fold decrease), *Ctsb* (0.5-fold decrease), *Ctsd* (0.7-fold decrease), *Ctss* (0.3-fold decrease), and *Ctsz* (0.2-fold decrease), was downregulated in soft tissue. Probe sets representing tissue inhibitor of

metalloproteinases *Timp1* (five-fold increase) and *Timp2* (three-fold increase) were modestly upregulated in calvarial bone and downregulated (*Timp1*, *Timp2*, *Timp4*) in soft tissue during *T. forsythia* infection.

Calvarial histology

The sham-infected control mice showed a lack of edema and minimal inflammation in the soft tissue over the calvaria at the site of injection (Fig. 6A). Calvarial soft tissue swelling occurred at the injection site within 24 h of the first injection and was increased at 48 and 72 h in almost all of the mice injected with *T. forsythia*, but not in the sham-infected control mice. The *T. forsythia*-infected mice did not show local abscesses, ulceration of the overlying skin, or any evidence of spread of infection to neighboring sites. Histological examination of the calvarial sections revealed significant edema and an intense mixed inflammatory cell infiltrate consisting of aggregates of polymorphonuclear leukocytes, lymphocytes, and macrophages (Fig. 6B). Notably, prominent and increased numbers of osteoclasts were seen at the suture area on the inner aspect of the calvaria compared with sham-infected controls (data not shown). An increase in the size of the bone marrow spaces as a result of increased endosteal bone resorption was also noted. Activated osteoclasts were found along the entire suture area corresponding to the areas of bone resorption (Fig. 6C). *Tannerella forsythia* cells were not seen in Gram-stained calvarial sections from mice with moderate soft tissue inflammatory infiltrates.

DISCUSSION

We used a complementary DNA microarray to study the gene transcriptional profiles of host soft tissue and calvarial bone during a localized acute infectious challenge with the oral pathogen *T. forsythia* in BALB/c mice. Diverse functional classes of genes were altered by *T. forsythia*, suggesting that numerous cellular processes were transcriptionally modulated during the course of the infection.

The breadth of functional categories and gene families that were altered in response to the localized infection in calvarial soft tissue included transcription, cell proliferation, cell cycle, transport, cell adhesion, stress, apoptosis, defense response, ECM, and cell differentiation, indicating that *T. forsythia* elicits a multitude of specific gene expression changes during infection. In bone, functional categories and gene families that were altered in response to the localized infection included cell proliferation, cell cycle, transcription, transport, defense and immune response, apoptosis, and ECM. Similar functional categories were also altered following infection with other periodontal pathogens, *P. gingivalis* (Meka *et al.*, 2010) and *Treponema denticola* (Bakthavatchalu *et al.*, 2010) indicating a commonality of the molecular interactions following infection of these pathogens with host soft tissues and bone.

Tannerella forsythia infection induced robust transcriptional changes in ECM proteins in both calvarial bone and soft tissue. For example, expression of the genes for matrix metalloproteinases 13, 9, 2 and 14, (*Mmp13*, *Mmp9*, *Mmp2*, *Mmp14*) was upregulated in calvarial bone, whereas expression of tissue inhibitor of metalloproteinase (*Timp1*, *Timp2*, *Timp3*) was modestly upregulated during *T. forsythia* infection. Similar levels of upregulation of the expression of *Mmp* genes and *Timp1*, *Timp2*, and *Timp3* genes were recorded in bone following *Tr. denticola* infection using this model (Bakthavatchalu *et al.*, 2010), whereas *P. gingivalis* had no effect on the expression of *Mmp* and *Timp* genes (Meka *et al.*, 2010). TIMPs may also serve as an early indicator of the acute-phase response. Furthermore, expression of several of the ECM proteins, including chondroadherin, procollagen types I, III, and V, fibronectin 1, fibromodulin, osteomodulin, and biglycan was elevated during *T. forsythia* infection as well as with *Tr. denticola* infection (Bakthavatchalu

et al., 2010) but not following *P. gingivalis* infection (Meka *et al.*, 2010). The changes we found in ECM protein transcriptional expression levels are in agreement with those induced robustly by *Escherichia coli* lipopolysaccharide inoculation in a canine model of oral infection (Higgins *et al.*, 2003). The composition of the ECM is known to impact multiple cellular activities including differentiation, proliferation, and motility. Periostin (gene *Postn*; increased four-fold and two-fold in bone and soft tissue, respectively), is a protein secreted into ECM and its gene is widely expressed in various tissues. The periostin gene is highly expressed in the embryonic periosteum, periodontal ligaments, and placenta among other tissues. Moreover, *Postn* null mice exhibit incisor enamel defects and an early-onset periodontal disease-like phenotype, suggesting that periostin is critically required for maintenance of the periodontal ligament (Rios *et al.*, 2005, 2008). Changes in periostin (ECM protein) expression in bone and tissue after *Tr. denticola* (Bakthavatchalu *et al.*, 2010) and *T. forsythia* challenge but not *P. gingivalis* infection (Meka *et al.*, 2010) in our model probably reflect its participation in the tissue remodeling of damaged calvarial bone and tissue after the acute *T. forsythia* or *Tr. denticola* infection. Damage caused by *P. gingivalis* infection, may be different from that induced by *T. forsythia* or *Tr. denticola*.

Tannerella forsythia has a number of virulence factors that can induce an inflammatory cascade involving proinflammatory cytokines, reactive oxygen species, and MMPs, and hence lead to the destruction of supportive soft and hard tissues around the teeth during periodontal disease. Several MMPs including MMP-2, MMP-3, MMP-8, and MMP-9 are increased in inflamed gingival tissue (Tervahartiala *et al.*, 2000), and increased levels of MMP-8 and MMP-9 have been reported in gingival crevicular fluid during the active stages of periodontal disease (Kiili *et al.*, 2002).

A significant difference between the calvarial bone and soft tissue samples was in the impact of infection on the LTM pathway in bone (33 genes increase; four genes decrease) including activation of Vav (signal transducer protein) which subsequently activates Rho-associated kinase, myosin light chain phosphorylation, resulting in the activation of actin (cytoskeleton) and α-cadherin. Moreover, leukocytes have a number of functions, including activation of endothelial cell signals, production of reactive oxygen species with subsequent activation of integrin β_2, platelet endothelial cell adhesion molecule-1 (PECAM1), MMPs, CDH5 (cadherin 5), and CAMs. This LTM pathway is consistent with the major functional pathways and several of the genes (integrins, focal adhesion molecules, cadherins) upregulated during induction of experimental gingivitis in humans, which is consistent with the activation of the LTM pathway (Offenbacher *et al.*, 2009). PECAM-1 is one of the most abundant proteins on the endothelial cell surface. It is expressed on the surface of platelets and leukocytes, and its expression on mononuclear infiltrates increases significantly with increasing size of infiltrates in the lesions of gingivitis and periodontitis (Gemmell *et al.*, 1994). In contrast, in tissue more than 18 LTM genes were downregulated and seven genes were activated indicating a lesser interaction of *T. forsythia* in the soft tissues. These differences (LTM upregulation in bone and downregulation in soft tissue) are likely related to the differences in the types of cells in both types of tissues, given the clear difference in the composition of both tissues, i.e. there was an acute inflammatory infiltrate in the soft tissue, but not in the underlying bone. Importantly, the number (impact factor: bone 85 and tissue 148) and pattern of genes (bone: 37/119; tissue: 35/119) altered in the LTM pathway of bone samples derived from *T. forsythia* infections are quite different from the gene expression after *Tr. denticola* challenge (bone: impact factor 306; genes 14/119) (Bakthavatchalu *et al.*, 2010). In contrast, the LTM pathway was not significantly affected in bone and soft tissue (impact factor: bone 18 and tissue 0) after *P. gingivalis* infection (Meka *et al.*, 2010). Further studies will be required to determine why there are differences between bone and soft tissue levels of expression of LTM pathway members in response to these three pathogens.

Another major difference between the bone and tissue samples was in the impact of *T. forsythia* infection on the CAM pathway. In calvarial bone, *T. forsythia* upregulated several components of this immune system pathway including major histocompatibility complex class II (MHCII), MHCI, intercellular adhesion molecule 1 (ICAM-1), ICAM-2, and ICAM-3, and downregulated T cell, cytotoxic T cell, and T helper–B cell interacting molecules. This is consistent with recent descriptions of the expression of class I and II MHC antigens on oral mucosal epithelium and induction of activation of $CD4^+$ T cells in response to bacterial challenge (Matsuyama *et al.*, 2005). Similarly, *Tr. denticola* and *P. gingivalis* upregulated the expression of a range of genes in this pathway including, MHCII, MHCI, CD8, CD28, CD80, and CD40 ligand (Bakthavatchalu *et al.*, 2010; Meka *et al.*, 2010). In contrast to *T. forsythia*, both *Tr. denticola* and *P. gingivalis* infections downregulated expression of ICAM-1, -2, and -3 in tissues (Bakthavatchalu *et al.*, 2010; Meka *et al.*, 2010). Increased expression of ICAM-1 (a marker of endothelial dysfunction) has been found in gingival tissues from patients with periodontitis (Hayashi *et al.*, 1994), and significant changes in soluble ICAM-1 levels of subjects with periodontitis were observed following treatment (Hannigan *et al.*, 2004). ICAM-1 is required for cell adhesion in inflamed tissues (Tamai *et al.*, 2005) and is also involved in the invasion of epithelial cells by *P. gingivalis* and *Treponema medium* (Tamai *et al.*, 2005, 2007). In contrast to *T. forsythia*, only MHCI was upregulated in soft tissue and T cell, cytotoxic T cell, and T helper–B cell interacting molecules were downregulated following *Tr. denticola* infection.

Tannerella forsythia upregulated several components of the ECM–receptor pathway in calvarial bone including collagen, chondroadherin, fibronectin, osteopontin, bone sialoprotein, and thrombospondin. These components of the ECM–receptor pathway were not regulated by either *Tr. denticola* (Bakthavatchalu *et al.*, 2010) or *P. gingivalis* infections (Meka *et al.*, 2010) in this model. Collagen-I and Collagen-III are the major constituents of all periodontal tissues and are essential in establishing their structural and physiological integrity. Fibronectin (critical in regenerating periodontal tissues), osteopontin, and bone sialoprotein are non-collagenous glycoproteins, seem to have a unique distribution within the periodontium and accumulate predominantly at the hard tissue interfaces. As *T. forsythia* levels are higher in subgingival plaque samples from deep periodontal pockets (Tanner & Izard, 2006), they may induce the expression of several MMPs and cathepsins, which collectively can degrade ECM proteins in the periodontium and may contribute to the bone resorption and attachment loss observed in periodontal disease (Hannas *et al.*, 2007). This level of *T. forsythia*-induced enhanced ECM–receptor interactions was not observed with infections by either *P. gingivalis* (Meka *et al.*, 2010) or *Tr. denticola* (Bakthavatchalu *et al.*, 2010). Similar observations in this pathway were not seen in the soft tissues after infection. These findings clearly suggest that *T. forsythia*, a potential critical member of pathogenic biofilms, may be an important contributor to the hard tissue destruction observed in periodontal disease.

The significant increase in osteoclast numbers and activity, dense inflammation, and full thickness calvarial bone resorption defects observed in response to *T. forsythia* infection were similar to the effects that we observed in the calvariae of mice following infection with other periodontal pathogens including *P. gingivalis* (Meka *et al.*, 2010), *Tr. denticola* (Bakthavatchalu *et al.*, 2010), *Campylobacter rectus* and *F. nucleatum* (Zubery *et al.*, 1998), as well as with local injections of IL-1 (Boyce *et al.*, 1989).

In conclusion, the present study reports findings from a comprehensive gene expression profile of inflamed soft tissue and calvarial bone that accompanied a localized, acute infection of mice with *T. forsythia*. Importantly, many of the most affected genes were related to biological processes, and specifically a large array of genes impacted ECM–receptor interaction in calvarial bone. Further studies are required to identify specific *T.*

forsythia virulence factors that alter host gene expression. Furthermore, the substantive impact of *T. forsythia* in upregulation of host inflammatory/innate immune responses in this model supports the need for additional studies to explore the role of selected genes in the periodontal disease process, as well as the effect of *T. forsythia* on host response patterns as a component of a polymicrobial infection in periodontal disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Arnold Stromberg, Cidambaram Srinivasan, Christopher Saunders, and Matt Hersh (Department of Statistics, University of Kentucky, Lexington, KY) for initial data management and microarray data analysis. We also thank Dr Quey-Chen and Ms Donna Wall (University of Kentucky, Lexington, KY; Microarray Core Facility) for microarray technical assistance. This study was supported by the National Institute of Dental and Craniofacial Research DE015720 to L.K., DE11111 to R.J.L., United States Public Health Services research grant U24 DE016509 (Robert Burne), and by a National Institute of Arthritis, Musculoskeletal and Skin Diseases grant (AR43510) to B.F.B.

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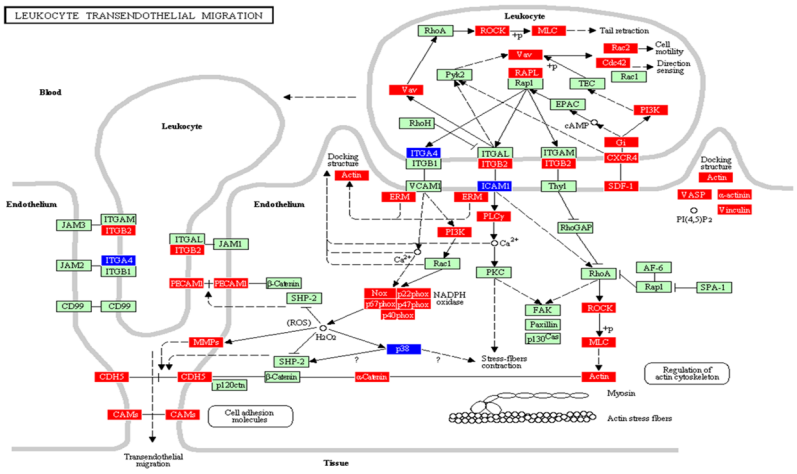


Figure 1.

Leukocyte transendothelial migration pathway containing genes differentially regulated by *Tannerella forsythia* in calvarial bone compared with sham-infected controls at $P \leq 0.05$, adapted from PATHWAY EXPRESS and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Genes shown in red are upregulated, genes shown in blue are downregulated, and green indicates no change in gene expression at the $P < 0.05$ significance level. +p, phosphorylation event; −p, dephosphorylation event; ?, receptors that are yet to be identified; O, other molecule. An arrow indicates a molecular interaction resulting in transendothelial migration, leukocyte activation, regulation of actin cytoskeleton and a line without an arrowhead indicates a molecular interaction resulting in inhibition.

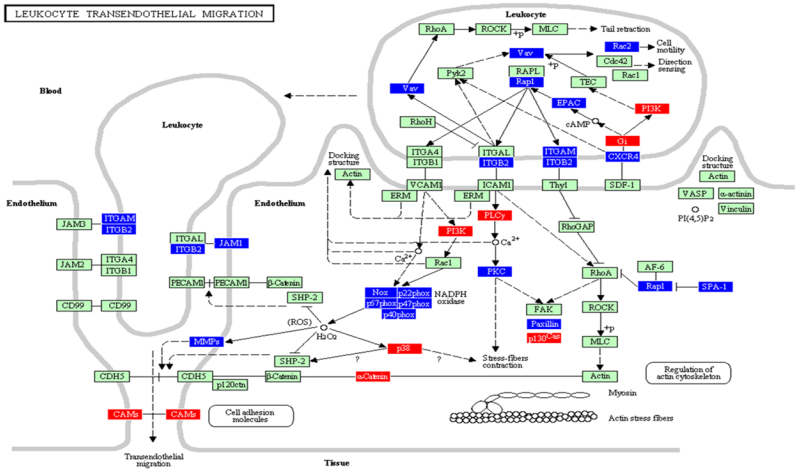


Figure 2.

Leukocyte transendothelial migration pathway containing genes differentially regulated by *Tannerella forsythia* in calvarial soft tissue compared with sham-infected controls at $P \leq 0.05$, adapted from PATHWAY EXPRESS and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Red indicates induction, blue indicates repression, and green indicates no change in gene expression. +p, phosphorylation event; −p, dephosphorylation event; ?, receptors that are yet to be identified; O, other molecule. An arrow indicates a molecular interaction resulting in transendothelial migration, leukocyte activation, regulation of actin cytoskeleton and a line without an arrowhead indicates a molecular interaction resulting in inhibition.

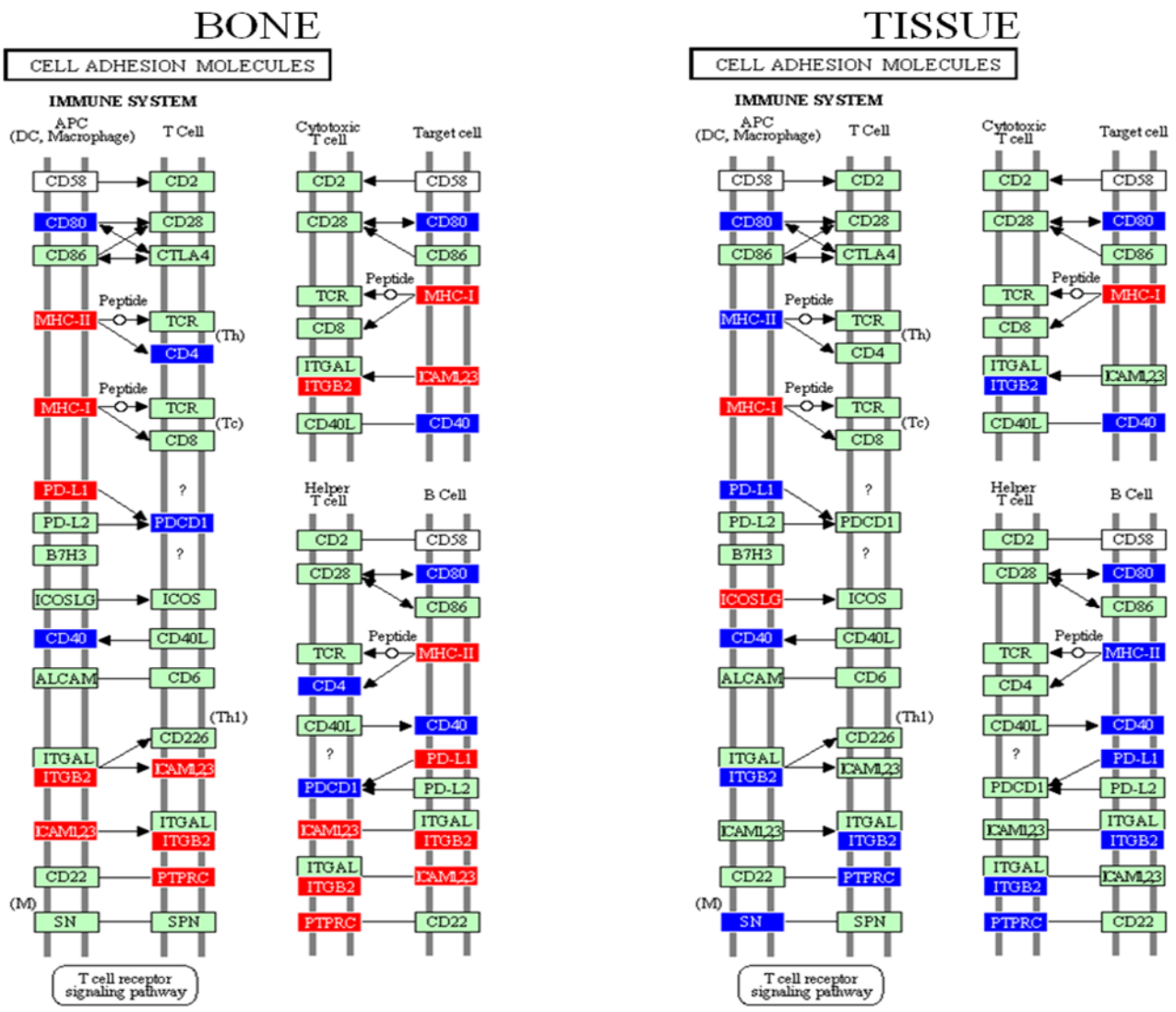


Figure 3.

Cell adhesion molecules pathway (immune system) containing genes differentially regulated by *Tannerella forsythia* in calvarial bone and soft tissue compared with sham-infected controls at $P \leq 0.05$, adapted from PATHWAY EXPRESS and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Red indicates induction, blue indicates repression, and green indicates no change in gene expression. An arrow indicates a molecular interaction resulting in activation of dendritic cells, macrophages, T cells (T-cell receptor signaling pathway), and a line without an arrowhead indicates a molecular interaction resulting in inhibition.

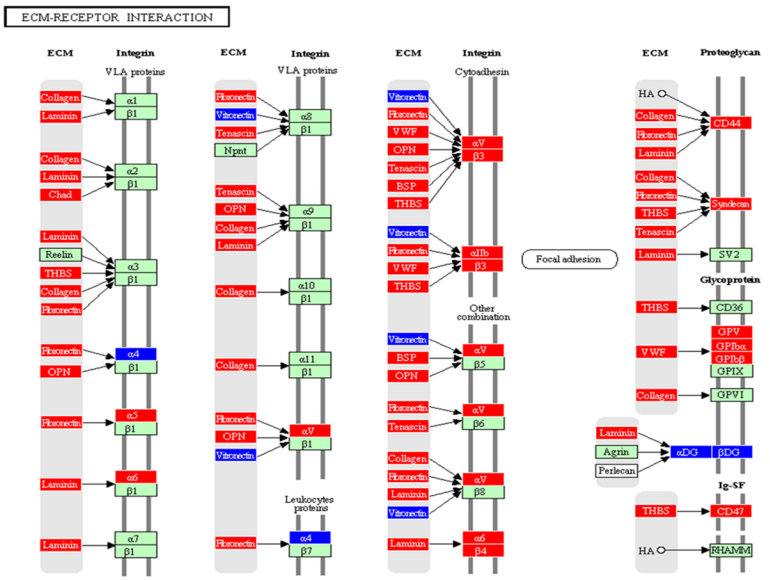


Figure 4.

Extracellular membrane receptor interaction pathway containing genes differentially regulated by *Tannerella forsythia* in calvarial bone compared with sham-infected controls at $P \leq 0.05$, adapted from PATHWAY EXPRESS and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Red indicates induction, blue indicates repression, and green indicates no change in gene expression. An arrow indicates a molecular interaction resulting in extracellular matrix receptor activation, regulation of integrin (VLA proteins, leukoproteins, cytoadhesin, focal adhesion, proteoglycan, glycoprotein).

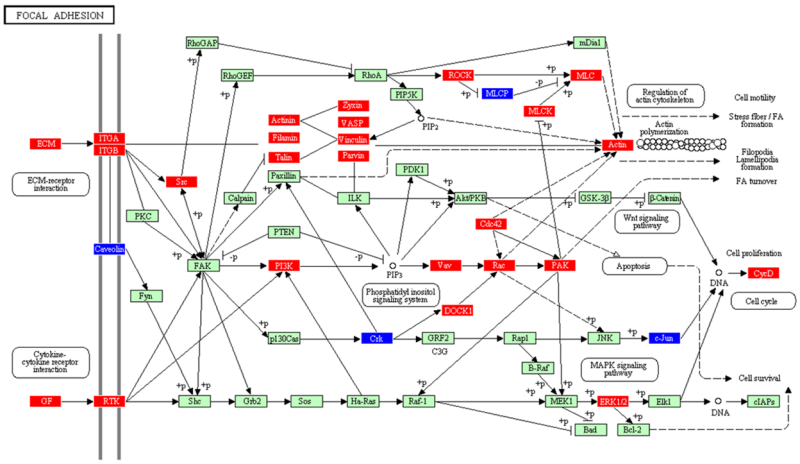
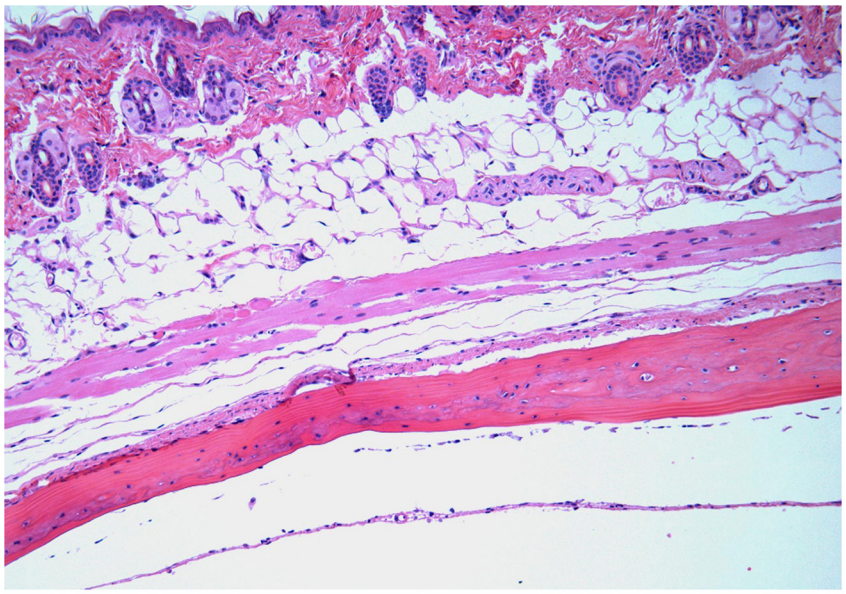
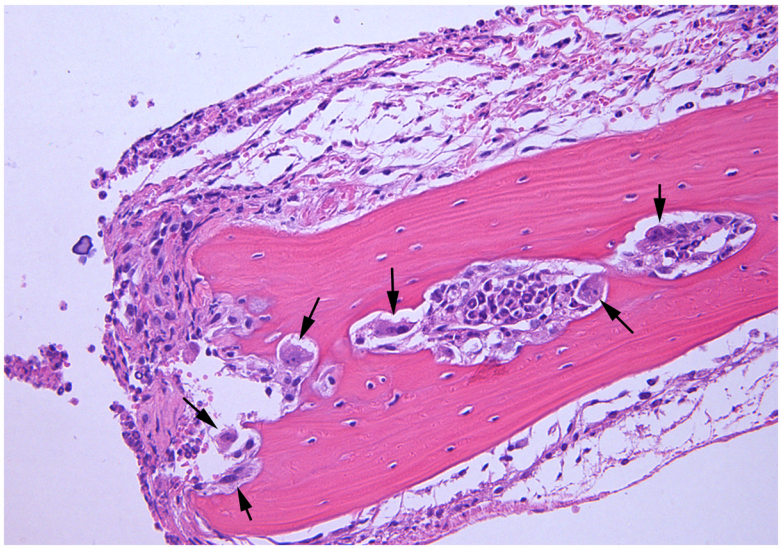


Figure 5.

Focal adhesion pathway containing genes differentially regulated by *Tannerella forsythia* in calvarial bone compared with sham-infected controls at $P \leq 0.05$, adapted from PATHWAY EXPRESS and using the Kyoto Encyclopedia of Genes and Genomes nomenclature. Red indicates induction, blue indicates repression, and green indicates no change in gene expression. An arrow indicates a molecular interaction resulting in extracellular matrix-receptor interaction, cytokine–cytokine receptor interaction, regulation of actin cytoskeleton, cell proliferation, cell cycle, and three signaling pathway systems (phosphatidyl inositol, mitogen-activated protein kinase, Wnt).





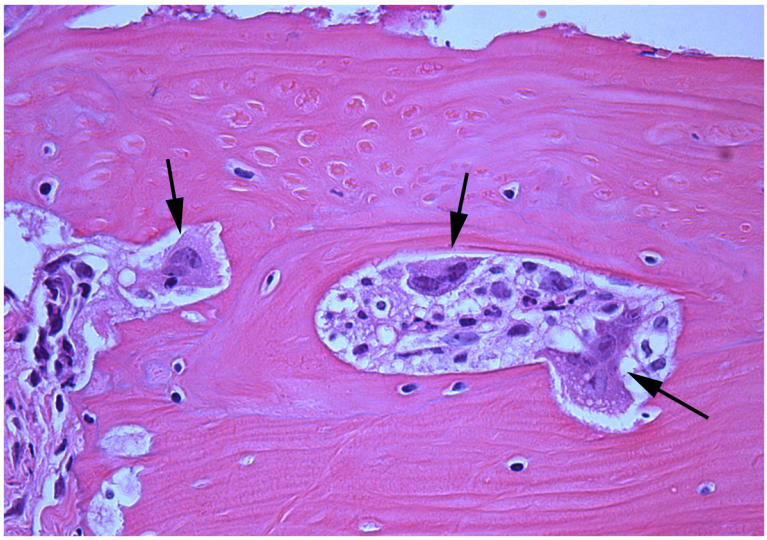


Figure 6.

Effects of *Tannerella forsythia* local injection on mouse calvaria. Live *T. forsythia* bacteria (1.5×10^9) were injected once daily for 3 days into the subcutaneous tissues overlying the calvaria of mice. All photomicrographs are of sections stained with hematoxylin & eosin. (A) Lack of edema and inflammation in the calvarial soft tissue of a sham-infected control mouse (10 ×). (B) Numerous osteoclasts (black arrows) are seen throughout the inner aspects of the calvarial bone mainly in the suture area. (20 ×). (C) Activated osteoclasts at higher magnification (40 ×). Also note the increase in marrow space size, as a result of the increased bone resorption in (B, C).

Table 1

Ontology analysis of calvarial bone and soft tissue gene pathways impacted by infection with *Tannerella forsythia*[1]

Impacted pathway[2]	Impact factor[3]	No. of input genes/no.of pathway genes[4]
Calvarial bone		
Leukocyte transendothelial migration	85.1	37/119
Cell adhesion molecules	79.3	30/159
ECM–receptor interaction	23.5	35/81
Focal adhesion	18.8	59/199
DNA replication	15.3	18/36
Phosphatidylinositol signaling system	14.5	14/75
Adherens junction	13.1	20/77
Hematopoietic cell lineage	11.7	29/86
Regulation of actin cytoskeleton	11.7	53/213
Cell cycle	9.7	32/124
B-cell receptor signaling pathway	9.1	19/71
Antigen processing and presentation	8.9	20/100
Natural killer cell-mediated cytotoxicity	5.7	22/109
Jak-STAT signaling pathway	5.7	31/157
Calvarial soft tissue		
Leukocyte transendothelial migration	148.2	35/119
Cell adhesion molecules	136.7	31/159
Adherens junction	42.7	19/77
Antigen processing and presentation	37.1	11/100
Phosphatidylinositol signaling system	16.0	18/75
Toll-like receptor signaling pathway	9.8	33/101
Natural killer cell-mediated cytotoxicity	9.8	31/109
B-cell receptor signaling pathway	9.4	23/71
Jak-STAT signaling pathway	8.9	41/157
Cytokine–cytokine receptor interaction	8.5	63/249
Focal adhesion	6.8	50/199
ErbB signaling pathway	5.9	24/89
p53 signaling pathway	5.5	21/74
Complement and coagulation cascades	5.4	21/74
VEGF signaling pathway	5.4	21/76

[1]The calvarial soft tissue and bone gene pathways were determined by PATHWAY EXPRESS (Draghici *et al.*, 2007; Khatri *et al.*, 2007).

[2]Kyoto Encyclopedia of genes and genome pathways (http://www.genome.jp/kegg/).

[3]The impact factor measures the pathways most affected by changes in gene expression in calvarial bone and soft tissue in response to *T. forsythia* by considering the proportion of differentially regulated genes, the perturbation factors of all pathway genes, and the propagation of these

perturbations throughout the pathway (Draghici *et al.*, 2007; Khatri *et al.*, 2007). Only pathways with an impact factor greater than 5 are included in this table.

[4]Number of regulated genes in a pathway/total number of genes currently mapped to this pathway.