Multiple Functions of the Leucine-Rich Repeat Protein LrrA of Treponema denticola

Akihiko Ikegami,1 Kiyonobu Honma,2 Ashu Sharma,1 and Howard K. Kuramitsu1*

Department of Oral Biology, School of Dental Medicine, State University of New York, Buffalo, New York 14214,1
and Department of Microbiology, Oral Health Science Center, Tokyo Dental College, Chiba 261-8502, Japan2

Received 27 October 2003/Returned for modification 31 December 2003/Accepted 26 April 2004

The gene lrrA, encoding a leucine-rich repeat protein, LrrA, that contains eight consensus tandem repeats of 23 amino acid residues, has been identified in Treponema denticola ATCC 35405. A leucine-rich repeat is a generally useful protein-binding motif, and proteins containing this repeat are typically involved in protein-protein interactions. Southern blot analysis demonstrated that T. denticola ATCC 35405 expresses the lrrA gene, but the gene was not identified in T. denticola ATCC 33520. In order to analyze the functions of LrrA in T. denticola, an lrrA-inactivated mutant of strain ATCC 35405 and an lrrA gene expression transformant of strain ATCC 33520 were constructed. Characterization of the mutant and transformant demonstrated that LrrA is associated with the extracytoplasmic fraction of T. denticola and expresses multifunctional properties. It was demonstrated that the attachment of strain ATCC 35405 to HEp-2 cell cultures and coaggregation with Tannerella forsythensis were attenuated by the lrrA mutation. In addition, an in vitro binding assay demonstrated specific binding of LrrA to a portion of the Tannerella forsythensis leucine-rich repeat protein, BspA, which is mediated by the N-terminal region of LrrA. It was also observed that the lrrA mutation caused a reduction of swarming in T. denticola ATCC 35405 and consequently attenuated tissue penetration. These results suggest that the leucine-rich repeat protein LrrA plays a role in the attachment and penetration of human epithelial cells and coaggregation with Tannerella forsythensis. These properties may play important roles in the virulence of T. denticola.

The oral spirochete Treponema denticola is strongly associated with the pathogenesis of periodontal diseases (3, 5, 19, 32, 41). This organism is frequently found in large numbers at diseased periodontal sites (45), and it expresses a variety of potential pathogenic properties, such as invasion of tissues, production of tissue-destructive enzymes, formation of cytotoxic products, and immunosuppression of host cell functions (3, 5, 19). The ability to attach to extracellular matrix molecules and/or bacterial partners may play an important role in the initiation of oral bacterial infections. The attachment of T. denticola to epithelial cells and extracellular matrix components was reported previously (1, 6, 14, 23–25, 38, 39, 48). In T. denticola, several surface proteins that mediate binding to proteins have been identified as putative virulence factors, i.e., fibronectin and collagen binding proteins (46, 47), Msp (8-10, 36), chymotrypsin-like protease complex (10), solute binding protein of an ATP-binding cassette-type peptide transporter, OppA (11), and hemin binding protein HbpA (49).

Leucine-rich repeats (LRRs) are protein interaction motifs of 20 to 29 residues consisting of a high proportion of leucine residues (20–22, 26, 27). These motifs are present in a large number of proteins with diverse functions and cellular locations in a variety of organisms and are mainly found in cell adhesion factors, hormone receptors, and enzyme inhibitors. Most of these proteins appear to be involved in protein-protein interactions. Recently, it was proposed that seven subfamilies of LRR proteins consist of different lengths and consensus sequences within the repeats (22, 27). In bacteria, at least three subfamilies of LRR extracellular proteins have been identified as bacterial, SDS22-like, and TpLRR (7, 27, 35). Specifically, the LRR proteins of the TpLRR family have been associated with bacterial cell surfaces, and these were identified as TpLRR from Treponema pallidum (44), BspA from Tannerella forsythensis (formerly Bacteroides forsythus) (43), and PepA from Streptococcus pneumoniae (42).

T. pallidum belongs to the same genus as T. denticola (3), and Tannerella forsythensis is known as a putative periodontal pathogen like T. denticola (15, 16). It has been suggested that TpLRR may facilitate interactions between components of the Treponema cell envelope (44) and BspA may play roles in adherence to oral tissues and triggering of the host immune responses (17, 43). However, the exact function of these cell surface-associated proteins of the TpLRR family is unknown.

In the present study we identified the leucine-rich repeat gene lrrA from T. denticola and constructed a homologous lrrA-inactivated mutant. In addition, the lrrA gene was expressed in another strain of T. denticola, ATCC 33520, which was shown to lack this gene. Examination of these constructs suggested several functions of the T. denticola LrrA which might be important in the virulence of these organisms.

**MATERIALS AND METHODS**

Bacterial strains and cultivation. T. denticola ATCC 35405 and ATCC 33520 were grown at 37°C under anaerobic conditions (85% N2, 10% H2, 5% CO2) in TYGV5 medium (37). Tannerella forsythensis ATCC 43037 and its BspA-defective mutant, BFM-571 (17), were grown in BF broth under anaerobic conditions (43). Porphyromonas gingivalis strain 381 and Fusobacterium nucleatum NCTC 11326 were grown in tryptic soy broth (Becton Dickinson and Co., Franklin Lakes, N.J.) supplemented with hemin (10 μg/ml) and menadione (5 μg/ml) under anaerobic conditions. Escherichia coli JM109 was used as a host strain for...
plasmid construction and expression of fusion proteins. Ampicillin was added to Luria broth (LB) (Becton Dickinson and Co.) as indicated.

Cloning and sequencing of the lrrA gene of T. denticola. The lrrA gene of T. denticola ATCC 35405 was amplified with oligonucleotide primers plrrAf (5'-AACTTTTGCCTAAGTGTGC-3') and plrrArm (5'-TATTTTTGGGAAACGGCAG-3'). The amplified fragment was cloned into the pCR2.1 vector (Invitrogen Corp., Carlsbad, Calif.). The resulting plasmid, pCRIIa, was cut with Accl within the lrrA fragment, and an erm-emr cassette from pTVZ198 was inserted into the Accl site of pCRIIa to construct plasmid pCRIa. Plasmid pCRIa was then linearized following EcoRV digestion, and 10 µl of linearized plasmid was used to transform T. denticola ATCC 35405 by electroporation as described previously (31). The erythromycin-resistant transformants were isolated and inoculated into TYGVS medium containing erythromycin (40 µg/ml).

Southern blot analysis. Chromosomal DNA of T. denticola was isolated and digested with PvuII. The digested DNA was separated on 1.0% agarose gels and transferred to Hybond-N+ membranes (Amersham Biosciences) by capillary transfer. The digoxigenin-labeled probe was the 0.31-kb fragment amplified by PCR with two synthetic oligonucleotide primers, pTA210f (5'-AACTTTTGCCTAAGTGTGC-3') and pTA520 (5'-AAGCATATCCGATTTG-3') from strain ATCC 35405. Digoxigenin labeling, hybridization, and detection were performed with a DIG DNA detection system (Roche Diagnostics Corporation, Indianapolis, Ind.) according to the manufacturer’s protocol.

Expression of the lrrA gene in T. denticola. The lrrA gene expression transformant of T. denticola ATCC 35405 was constructed with a T. denticola shuttle vector system (4). The lrrA gene containing an upstream region including the Shine-Dalgarno sequence was amplified by PCR with primers pKMAf (5'-TTTGTTCACATCTTCTGAATC-3') and pKMAR (5'-TTTATTGAGAGCGAGTATG-3'). The DAlr sequence was amplified by PCR with primers pKMAf and BglII recognition sites were added (shown in lowercase letters), was inserted into the SacI and BglII sites of shuttle vector pMKR4PE. The resulting plasmid, pKMIRa, was transformed into T. denticola ATCC 35420. The transformant was examined for expression of the LrrA protein by Western blot analysis.

Overexpression and purification of the LrrA protein. The different regions of the PCR-amplified lrrA gene to which BamHI and PstI recognition sites had been added were cloned between the BamHI and PstI sites of a high-level expression hexahistidine-tagged recombinant protein vector pEBO8 (Qiagen Inc., Valencia, Calif.). After verification of the resultant nucleotide sequences, the plasmids encoding the different regions of hexahistidine-tagged modified LrrA proteins were used for expression of the LrrA proteins (His-LrrA, His-LrrAAc, His-LrrAL and His-LrrALAc). Cells of E. coli XL1-Blue MRF were transformed carrying these plasmids were grown in LB medium. Expressions of the chimeric genes was induced by addition of 1 mM isopropylthiogalactoside (IPTG), and the recombinant proteins were purified by Ni2+ -nitrotitractric acid spin affinity column chromatography (Qiagen Inc.) and Hitrap trapping HP column chromatography from the HisTrap kit (Amersham Biosciences). Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% PAGE) and visualized by staining with Coomassie brilliant blue R.

Preparation of anti-LrrA antibody. The recombinant protein His-LrrALAc was separated by SDS-12% PAGE and visualized by staining with Coomassie brilliant blue R. A gel strip containing the His-LrrALAc protein band was used as an antigen. Antiserum containing polyclonal antibodies against His-LrrALAc (anti-LrrA serum) was obtained by injecting the antigen into chickens with custom anti-LrrA antibodies. Proteins were clarified by centrifugation at 16,000 g for 2 h at room temperature, followed by 1:10,000-diluted horseradish peroxidase-conjugated goat anti-chicken immunoglobulin Y (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) for 1 h at room temperature and detected by a horseradish peroxidase-ide assay kit (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.).

Immunogold labeling and observations by transmission electron microscopy. Immunogold labeling was performed by the modified method of Ishihara et al. (38). Briefly, cells of Escherichia coli cells were harvested by centrifuging at 3,000 x g for 10 min and washed with phosphate-buffered saline (PBS) twice. Bacterial cells were then adjusted to an optical density at 600 nm of 0.5. Bacterial cell suspensions were next reacted with 10-fold-diluted anti-LrrA serum on 200-mesh collagen membranes (Nishin EM Co. Ltd., Tokyo, Japan) at 37°C for 30 min. After primary antibody reactions, membranes were washed with PBS twice, and the membranes were reacted with anti-chicken IgG rabbit serum conjugated to 10-fold-diluted gold particles (10 nm; Invitrogen Corp., Carlsbad, United Kingdom) at 37°C for 30 min. After reaction, the membranes were washed twice with PBS. The membranes were then negatively stained with 4% uranyl acetate solution. Four samples were made for each specimen. Observations were made with an AEM H-7100 transmission electron microscope (Hitachi Co., Ltd., Tokyo, Japan).

Attachment of T. denticola to HEP-2 epithelial cells. HEP-2 epithelial cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen Corp.) supplemented with fetal bovine serum (Invitrogen Corp.) under 5% CO2 at 37°C. After the HEP-2 cells formed a tissue layer, the medium was changed to unsupplemented DMEM for the experiments. T. denticola strains were harvested and resuspended in unsupplemented DMEM to a density of 5 x 106 cells/ml. The bacterial suspensions (106 cells) were added to the HEP-2 tissue layers and incubated for 1 h at 37°C. After incubation, the tissue layers were washed twice with PBS containing 0.5% Tween 20 (PBST) to remove nonadhering bacteria. The tissue layers were then incubated with anti-LrrA IgG serum conjugated to 10-fold-diluted gold particles (10 nm) (100 µl of serum in PBS) for 1 h at room temperature. The percent coaggregation was determined over time at room temperature. The percent coaggregation was calculated by the following equation: coaggregation (%) = [preincubation value [OD600] - sample value [OD600]]/preincubation value [OD600] x 100.

Binding assays of the LrrA derivatives to the recombinant Bsp70 protein. The wells of 96-well microtiter plates (Invitrogen Corp.) were coated with 2 µg of recombinant Bsp70 (amino acid residues 17 to 724 of BspA) (43) dissolved in PBS followed overnight incubation at 4°C. The protein-coated wells were then blocked with 0.5% bovine serum albumin in PBS for 1 h at room temperature. Various concentrations (1 and 10 pmol) of the His-tagged LrrA derivatives in 0.2% bovine serum albumin–PBS were prepared for binding assays. The proteins were added to the pretreated wells and incubated for 1 h at 37°C, followed by washing three times with PBST. The bound proteins were detected by QAE-anchored IgG antibody to saturate His-LrrA protein conjugate (Qiagen Inc.) according to the supplier’s instructions. The anti-His-rose- radis porse conjugate was dialyzed into 1:1,000 and developed with Tris- maleate buffer (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

Swarming assay. T. denticola was incubated in TYGVS medium swim plates containing 0.8% SeaPlaque agar (FMC BioProducts, Rockland, Maine) at 37°C aerobically, and swarming was monitored visually.

Tissue penetration assay. Tissue penetration was determined by a modification of the penetration assay of Lux et al. (33). Tight-junctioned HEP-2 (now recognized as HeLa contaminants) tissue layers were incubated in supplemented DMEM in 3.0-µm Falcon cell culture inserts (Becton Dickinson Co.) under the culture conditions used for the attachment assays. T. denticola strains were harvested and resuspended in unsupplemented DMEM to a density of 5 x 106 cells/ml. The suspensions (2.5 x 106 cells) were added to the tissue layers and incubated for 8 h under anaerobic conditions. Penetration rates were determined by counting cell numbers with a counting chamber (Hauser Scientific Partnership, Horsham, Pa.). The resistance of tissue layers was measured directly by a transmission electron microscope (Hiten Chi Co., Ltd., Tokyo, Japan).

Received for publication September 22, 2017.Accepted October 9, 2017.
RESULTS

Cloning and sequencing of the lrrA gene. The LRR sequence in T. denticola was identified in the genome database of T. denticola ATCC 35405 from TIGR with the BspA sequence. As a result, a gene encoding a highly conserved LRR sequence was found in the database in addition to several other potential LRR protein genes. One of these genes was then isolated to confirm its nucleotide sequence (Fig. 1). After verification of the nucleotide sequence, the full-length gene, consisting of 1,068 bp, was named lrrA (leucine-rich repeat A). The lrrA gene was found to encode a putative protein consisting of 355 amino acid residues with a predicted molecular mass of 37,231 Da. A putative Shine-Dalgarno sequence was also identified in the sequence. Although the TpLRR protein from T. pallidum may not be a true lipoprotein, a potential signal peptidase cleavage site was identified in LrrA along with a consensus spirochetal lipoprotein box (13). This suggested that the LrrA protein may be a lipoprotein with a predicted molecular mass of 32,829 Da for the mature protein.

The amino acid sequence of the LRR motifs of LrrA show high homology to other LRR sequences, including BspA of Tannerella forsythensis (57% identity), TpLRR of T. pallidum (35% identity), and PcpA of Streptococcus pneumoniae (28% identity) by BlastP 2.2.6. Analysis of the amino acid sequence of LrrA also revealed that LrrA contains eight tandem repeats of 23 amino acid residues with the consensus pattern LTSIxLPANLTTIGEYAFxGCTG (Fig. 1), where x is any residue, and is analogous to a consensus sequence pattern from the TplLRR family of LRR proteins, LxxLxLxxxLxxIgxxAFxxC/Nxx (22, 27). Therefore, LrrA shares a conserved pattern of LRRs similar to those of the TplLRR family.

Construction of the lrrA mutant and pKMlrrA transformant. To determine the function of LrrA in T. denticola, an lrrA gene-defective mutant was constructed by allelic mutagenesis. A 905-bp portion of the lrrA gene was cloned into vector pCR2.1, and a 2.4-kb ermF-ermAM cassette was inserted into the AccI site of the lrrA gene to construct plasmid plrrAerm. Plasmid plrrAerm was then linearized with EcoRV and electroporated into T. denticola ATCC 35405. An insertion of the ermF-ermAM cassette into the lrrA gene on the chromosome by double crossover results in erythromycin-resistant (Ermr) transformants (Fig. 2A). The gene insertion in the transformants was confirmed by Southern blot analysis with a 311-bp fragment of the lrrA gene as a probe (Fig. 3). A 1.2-kb band was observed in strain ATCC 35405 (lane 2), while a larger 3.6-kb band was observed in the Ermr transformant AK-1 (lane 3). By contrast, no positive band was detected for strain ATCC 33520 (lane 1), indicating that a gene highly homologous to lrrA is absent in this strain. Therefore, to further analyze the function of LrrA in T. denticola, an lrrA gene expression plasmid was introduced into strain ATCC 33520 with a T. denticola shuttle vector system (4). The lrrA gene containing its Shine-Dalgarno sequence was cloned into T. denticola shuttle vector pKMR4PE (Fig. 2B). The resulting plasmid, pKMlrrA, was then transformed into strain ATCC 33520.
transformant was confirmed for expression of LrrA by Western blot analysis with anti-LrrA serum (see Fig. 5).

**Overexpression and purification of LrrA.** In order to prepare anti-LrrA antibody and identify the functional motifs of LrrA, various fragments of LrrA were expressed as fusion proteins with His tags in *E. coli* with the high-level expression system vector pQE80 (Fig. 4A). The fusion proteins included a full-length LrrA containing eight LRRs from residues 1 to 355 (His-LrrA), a C-terminal deletion LrrA containing four LRRs from residues 1 to 201 (His-LrrA/ΔC), an N-terminal deletion LrrA containing eight LRRs from residues 91 to 355 (His-LrrA/ΔN), and both N- and C-terminally deleted LrrA containing four LRRs from residues 91 to 201 (His-LrrAΔNC). These recombinant proteins were purified by affinity column chromatography and analyzed by SDS–15% PAGE. The results of SDS-PAGE analysis indicated that the recombinant proteins were expressed in sizes similar to the predicted molecular masses (His-LrrA, 38.6 kDa; His-LrrA/ΔC, 22.1 kDa; His-LrrA/ΔN, 29.5 kDa; and His-LrrAΔNC, 12.9 kDa). The recombinant proteins were purified as described earlier, and His-LrrAΔN was used for preparation of the anti-LrrA antibody.

**Expression of *T. denticola* LrrA protein in *T. denticola*.** Proteins were extracted from various *T. denticola* strains and fractionated by SDS–12% PAGE. Positive bands reacting with anti-LrrA serum were detected for strain ATCC 35405, the *lrrA::erm* mutant AK-1, and strain ATCC 33520. Chromosomal DNAs from strain ATCC 33520 (lane 1), strain ATCC 35405 (lane 2), and mutant AK-1 (lane 3) were digested with *Pvu*I. The digoxigenin-labeled probe was the 0.31-kb *lrrA* fragment from strain ATCC 35405. Digoxigenin-labeled DNA molecular size markers (in kilobases) are shown in lane 4.

**LrrA is associated with the cell surface of *T. denticola*.** Since the sequencing data suggested that LrrA may be a lipoprotein associated with the extracytoplasmic fraction of strain ATCC 35405, immunoelectron microscopy was performed to localize LrrA in strain ATCC 35405. As shown in Fig. 6A, cells of strain ATCC 35405 incubated with anti-LrrA serum displayed gold.
particles associated with the cell surface of the organism. However, labeling was not observed in the lrrA mutant AK-1 (Fig. 6B). These results demonstrated that the LrrA protein is a component of the extracytoplasmic cell fraction of T. denticola, consistent with its identity as a lipoprotein.

**Attachment of T. denticola to HEp-2 cell tissue layers.** To investigate the attachment of T. denticola to epithelial cells, attachment assays were performed with HEp-2 cell cultures.

Attachment to HEp-2 cell tissue layers was measured for four different T. denticola strains, ATCC 35405, AK-1, ATCC 33520, and ATCC 33520/pKMlrrA. The chymotrypsin specific activities of these strains as a percentage of that of strain ATCC 35405 were 79.8 ± 6.0% for mutant AK-1, 12.6 ± 0.7% for strain ATCC 33520, and 16.7 ± 3.5% for ATCC 33520/pKMlrrA.

The attachment of mutant AK-1 to HEp-2 cell tissue layers was reduced by 47.9% compared to that of parental strain ATCC 35405 (Fig. 7). Strain ATCC 33520 demonstrated weaker attachment (54.6%) in comparison with the LrrA-bearing strain ATCC 35405. Moreover, the attachment of ATCC 33520/pKMlrrA was slightly enhanced (81.6%) in comparison to its parental strain ATCC 33520. These results suggested that LrrA plays a role in the attachment of T. denticola to epithelial cells.

**Coaggregation activity between T. denticola and heterologous bacteria.** To analyze the role of LrrA in the coaggregation between T. denticola and heterologous oral bacteria, coaggregation activities were determined with T. denticola strains ATCC 35405, AK-1, ATCC 33520, and ATCC 33520/pKMlrrA.

Coaggregations between T. denticola mutant AK-1 and Porphyromonas gingivalis or Fusarium nucleatum were not significantly different compared to that of parental strain ATCC 35405 (Fig. 8A). By contrast, mutant AK-1 was attenuated in coaggregation with Tannerella forsythensis (Fig. 8B). In addition, a reduction in coaggregation was likewise observed between T. denticola ATCC 35405 and the Tannerella forsythensis BspA mutant BFM-571 (Fig. 8B). Interestingly, strain ATCC 33520 appeared to aggregate with Tannerella forsythensis at a slower rate than strain ATCC 35405. Moreover, ATCC 33520/pKMlrrA was increased in coaggregation with Tannerella forsythensis relative to its parental strain ATCC 33520 (Fig. 8C). These results, taken together, suggested that the coaggregation between T. denticola and Tannerella forsythensis is mediated, in part, by interaction of the LrrA protein with the corresponding LRR protein, BspA, of Tannerella forsythensis. Neither T. denticola nor Tannerella forsythensis exhibited autoaggregation under these conditions.
Binding assays of LrrA derivatives to recombinant Bsp70.

Since mutant analysis suggested that T. denticola-Tannerella forsythensis interactions may be mediated by LrrA-BspA binding, the interaction of the LrrA derivatives with BspA was analyzed directly. The binding assays demonstrated that the specific binding activity of the LrrA derivatives to recombinant Bsp70 was 104.2 ± 11.4% for His-LrrA, 45.2 ± 13.2% for His-LrpAΔC, 53.6 ± 13.4% for His-LrpAΔN, and 53.6 ± 13.4% for His-LrpAΔNC compared with His-LrrA (Fig. 9). These results suggested that the specific binding of LrrA to recombinant Bsp70 is mediated through the N-terminal region of LrrA, which is devoid of the LRR sequences.

Swarming activity of mutant AK-1 and transformant ATCC 33520/pKMlrrA.

The growth rates of mutant AK-1 and ATCC 33520/pKMlrrA were similar to that of the parental strains in TYGVS medium (data not shown). These strains and the parental strains also appeared to exhibit similar motility in liquid culture when observed by dark-field microscopy. However, mutant AK-1 was shown to be deficient in swarming on TYGVS swarm plates (containing 0.8% SeaPlaque agarose) (Fig. 10). Surprisingly, the ATCC 33520/pKMlrrA construct was also decreased in swarming compared to its parental strain ATCC 33520 without pKMlrrA or with the shuttle vector plasmid pKMR4PE alone. These results indicated that mutant AK-1 and ATCC 33520/pKMlrrA were reduced in swarming activities in high-viscosity medium. These data suggest that the swarming activity of T. denticola can be influenced by the presence or absence of LrrA in the spirochetes.

FIG. 8. Coaggregation between T. denticola and heterologous bacteria. (A) Coaggregation between T. denticola and heterologous bacteria. The percent coaggregation was calculated as described in the text. The data are the averaged values from three separate experiments. Td35405, T. denticola ATCC 35405; TdAK-1, T. denticola LrrA-deficient mutant AK-1; Tf, Tannerella forsythensis 43037; Pg, P. gingivalis 381; Fn, F. nucleatum 11326. (B) Effects of LRR proteins (LrrA and BspA) on coaggregation between T. denticola and Tannerella forsythensis, TdBFM571, Tannerella forsythensis BspA-deficient mutant BFM-571. (C) Effects of LrrA expression on coaggregation between T. denticola and Tannerella forsythensis. Td33520, T. denticola ATCC 33520; ATCC 33520/pKMlrrA, LrrA expression transformant of ATCC 33520.

FIG. 9. Binding of the LrrA derivatives to BspA (recombinant Bsp70). Binding activities of the various LrrA derivatives were determined by enzyme-linked immunosorbent assay. Microtiter plates coated with recombinant Bsp70 were incubated with the LrrA derivatives His-LrrA, His-LrpAΔC, His-LrpAΔN, and His-LrpAΔNC for 1 h at room temperature. Bound proteins were incubated with 1:1,000-diluted anti-His-horseradish peroxidase conjugate and developed with TMB. Binding values are indicated relative to binding of His-LrrA, which was set at 100%. Values are means and standard deviations (error bars) for three separate experiments performed in duplicate.

FIG. 10. Swarming activity of T. denticola. T. denticola strains ATCC 35405, AK-1, ATCC 33520, and ATCC 33520/pKMlrrA cultured in TYGVS medium swarm plates (containing 0.8% agarose) were monitored visually after 7 days.
Leucine-rich repeat proteins (LRRs) have been reported to be generally useful as protein-binding motifs (20–22, 26, 27). The major function of LRRs may be to provide a structural framework for the formation of protein-protein interactions (27). In this study, an lrrA gene identified in the genome database of T. denticola ATCC 35405 was used to analyze the function of LRR proteins in T. denticola. The lrrA gene was characterized as encoding a polypeptide of 355 amino acid residues that contains eight tandem repeats of 23 amino acid residues and exhibits high homology in the LRR region with other LRR proteins, such as BspA and TpLRR. Recently, the TpLRR family has been proposed as one of the subfamilies of LRR proteins (22). BspA and TpLRR belong to the TpLRR family, exhibiting consensus pattern LxxLxxLxxLxxxAxxxFxxxC/Nxx. The consensus pattern of the LRR in LrrA was identical to that of the TpLRR family. Analysis of the functions of the TpLRR family has suggested that TpLRR proteins may facilitate interactions of components of the cell envelope with the environment (44). Furthermore, BspA may play roles in adherence to oral tissues and triggering of host immune responses (17, 43). However, only two genes of the TpLRR family have been analyzed until now, and it was not clear what role LRR proteins had in T. denticola.

Southern blot analysis demonstrated that T. denticola ATCC 35405 contains an lrrA gene, while strain ATCC 33520 does not. Expression of the LrrA protein in strain ATCC 35405 as well as in strain ATCC 33520 transformed with the lrrA gene was confirmed by Western blot analysis with anti-LrrA serum. Furthermore, the data from immunoelectron microscopy suggested that LrrA is associated with the cell surface of T. denticola, although the precise location has not yet been determined. LRR proteins of the TpLRR family have also been reported to be cell surface associated (22). Specifically, it has been proposed that BspA may be concerned with cellular adhesion by Tannerella forsythensis (17, 43). The association of LrrA with the surface of T. denticola is also consistent with the observation from this study that LrrA plays a role in interaction of this spirochete with its oral environment.

The ability to attach to extracellular matrix molecules as well as bacterial partners may play an important role in the initiation of bacterial infection. T. denticola has been reported to attach to epithelial cells and extracellular matrix components (1, 6, 14, 23–25, 38, 39, 48). The present results regarding the attachment of T. denticola to HEp-2 cell tissue layers indicated that the LrrA protein plays a role in these interactions. Therefore, LrrA may be involved in the attachment of the spirochete to the epithelial cell layer lining the gingival crevice.

Coaggregation is a process by which bacteria attach to one another via specific cell surface molecules (40). These interactions are believed to contribute to the development of multispecies biofilms (40); therefore, coaggregation has important implications for the bacteria isolated from dental plaque biofilms. Many different coaggregation interactions have now been identified between oral bacteria (29, 30), and that of T. denticola has been reported to be influenced by P. gingivalis and F. nucleatum (12, 28). Some of the coaggregation adhesion proteins are multifunctional and can often interact with host oral surfaces as well as host proteins (40). Therefore, it is likely that LrrA may also be multifunctional and play a role in multiple interactions. The influence of LrrA on the coaggregation between T. denticola and heterologous bacteria indicated that LrrA did not affect coaggregations with P. gingivalis and F. nucleatum. However, coaggregation between T. denticola and Tannerella forsythensis was influenced by the LRR proteins LrrA and BspA. These results suggested that the coaggregation between T. denticola and Tannerella forsythensis is mediated, in part, by interaction of LrrA with BspA.

In vitro binding assays of the LrrA derivatives suggested that specific binding of LrrA to recombinant Bsp70 is not mediated by the interaction of the LRR domains but is mainly mediated through the N-terminal region of LrrA. Most LRR proteins have been previously reported to contain flanking regions that are an integral part of the LRR domain (2, 27). The LRR-associated motif LRR cap was identified in flanking regions.
and proposed to play a role in shielding the hydrophobic core of the LRRs from solvents (2, 27). In the case of LrrA, the LRR cap motif was not identified; however, a functional motif similar to an LRR cap may exist in the N-terminal region and could interact with the LRR domain of BspA. More detailed investigations will be necessary to identify the motifs involved in LrrA-BspA interactions.

Analysis of swarming indicated that the affected LrrA expression strains, mutant AK-1 and ATCC 33520/pKPtrrA, exhibited decreased swarming on TYGV agar plates, even though each appeared to be as motile as the parental strains in liquid cultures. This change may also attenuate tissue penetration, as demonstrated in the present study, because previous studies reported that the tissue penetration of oral epithelial cell layers by *T. denticola* is likely dependent upon motility and chemotaxis (33, 34). The attenuated swarming by AK-1 suggested that the LrrA protein is involved in the swarming ability of strain ATCC 35405. However, strain ATCC 33520 swarms as well as strain ATCC 35405 without expression of an LrrA protein. This suggests that the expression of LrrA in strain ATCC 33520 “interferes” with the swarming mechanism of this strain. Perhaps the abnormal expression and localization of LrrA in strain ATCC 33520 interferes with the cell surface properties of this strain that are required for swarming. In addition, since dentilisin activity may itself play a role in the surface properties of *T. denticola*, it is possible that interactions between the protease and LrrA could be involved in some of the results demonstrated in this study. For example, the relatively low protease activity of strain ATCC 35405 relative to that of strain ATCC 33540 could influence such interactions and alter surface properties. Additional approaches are ongoing to determine the molecular basis for these novel observations.

In the present study, it was demonstrated that the functions of the cell surface-associated LRR protein LrrA in *T. denticola* are consistent with a significant role for this protein in virulence. The protein appears to play a role in both attachment to epithelial cells and coaggregation with other periodontal pathogenic bacteria such as *Tannerella forsythensis*. Both of these interactions may be important in the colonization of *T. denticola* into subgingival plaque. Furthermore, a role for LrrA in tissue penetration was demonstrated, and this property may also be an important virulence factor for oral spirochetes.


