Adhesion characteristics of Listeria adhesion protein (LAP)-expressing Escherichia coli to Caco-2 cells and of recombinant LAP to eukaryotic receptor Hsp60 as examined in a surface plasmon resonance sensor

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Listeria adhesion protein; alcohol acetaldehyde dehydrogenase; Listeria monocytogenes; Hsp60; adhesion kinetics; SPR.

Abstract
Listeria adhesion protein (LAP) is an important adhesion factor in Listeria monocytogenes and interacts with its cognate receptor, mammalian heat shock protein 60 (Hsp60). The genetic identity of LAP was determined to be alcohol acetaldehyde dehydrogenase (Aad). A recombinant Escherichia coli strain expressing aad confirmed the involvement of Aad in adhesion to Caco-2 cells. Binding kinetics ($k_a$) of recombinant LAP (rLAP) to Hsp60 was examined in a surface plasmon resonance sensor and was determined to be $5.35 \times 10^9$ M$^{-1}$s$^{-1}$ and it was equivalent to the binding of anti-Hsp60 antibody ($k_a = 2.15 \times 10^8$ M$^{-1}$s$^{-1}$) to Hsp60. In contrast, Internalin B, an adhesion/invasion protein from L. monocytogenes, used as a control, had binding kinetics ($k_a$) of only $2.9 \times 10^7$ M$^{-1}$s$^{-1}$. The $K_D$ value of rLAP was $1.68 \times 10^{-8}$ M, which was significantly lower than Internalin B ($K_D = 6.5 \times 10^{-8}$ M). These results suggest that Hsp60 has significantly higher avidity for anti-Hsp60 antibody and LAP than Internalin B. In summary, LAP is identified as an alcohol acetaldehyde dehydrogenase and binding of recombinant E. coli to Caco-2 cells or rLAP to Hsp60 protein was found to be highly specific.

Introduction
Listeria monocytogenes is a human pathogen that carries a unique set of virulence gene clustered in a 9 kb pathogenicity island, including: prfA (positive regulatory factor), plc (phospholipase C), hlyA (hemolysin), mpl (metalloprotease), and actA (actin polymerization protein) (Vazquez-Boland et al., 2001). Adhesion and invasion associated genes located outside the pathogenicity island include internalins A and B (Cossart & Sansonetti, 2004), p60 or cell wall hydrolase (CwhA) (Park et al., 2000; Pilgrim et al., 2003), autolysin amidase (Ami) (Braun et al., 1997; Milohanic et al., 2000), fibronectin binding protein (Fbp) (Gilot et al., 2000; Dramsi et al., 2004), lipoteichoic acid (LTA) (Abachin et al., 2000; Pilgrim et al., 2003), and lipoprotein promoting entry protein (LpeA) (Reglier-Poupet et al., 2003). Adhesion and invasion are crucial in initiating L. monocytogenes infection in intestinal, utero-placental, hepatic, or neurological phase of infection.

Previously, we reported a 104 kDa Listeria adhesion protein (LAP) as an adhesion factor in L. monocytogenes (Pandiripally et al., 1999; Kim, 2004). Expression of LAP is primarily cytosolic, with some surface representation (Jaradat & Bhunia, 2002), and influenced by growth temperature (Santiago et al., 1999) and nutrient-limiting conditions (Jaradat & Bhunia, 2002). In vitro modeling demonstrated elevated LAP adhesion in cells derived from the ileocecal intestinal region (Jaradat et al., 2003) and
identified heat shock protein 60 (Hsp60) as the mammalian cell receptor (Wampler et al., 2004).

Listeria adhesion protein has been established as an important adhesion factor for L. monocytogenes pathogenesis (Jaradat et al., 2003); however, its genetic characteristic and binding kinetics to its receptor, Hsp60, are unknown. This information will help clarify the nature of LAP-mediated binding kinetics to its receptor, Hsp60, are unknown. This study, the gene encoding LAP was identified and its function was confirmed in a recombinant Escherichia coli strain. Binding kinetics of recombinant LAP with Hsp60 was investigated by surface plasmon resonance (SPR).

Materials and methods

Bacterial strains, plasmids, and media

Bacterial strains used in this study are listed in Table 1. Listeria monocytogenes and Escherichia coli were routinely cultured in brain heart infusion (BHI; Difco Laboratories, Sparks, MD) and Luria–Bertani (LB, 1% NaCl, 1% tryptone peptone, 0.5% yeast extract) media, respectively. Cultures were grown at 37°C with shaking at 150 rpm in a shaker incubator (New Brunswick Scientific, Edison, NJ). When needed, ampicillin (50 μg mL⁻¹) was added to E. coli culture. pGEMT-Easy Vector (Promega, Madison, WI) was used for cloning the lap (aad) gene from L. monocytogenes F4244. lap-negative transposon (Tn916) mutant L. monocytogenes A572 was grown in BHI containing tetracycline (10 μg mL⁻¹) (Pandiripally et al., 1999). Internalin B (InlB) containing E. coli strain BL21 (DE) with pET28b-1 was obtained from Pascale Cossart (Institut Pasteur, France) and was grown overnight with shaking in LB with 30 μg mL⁻¹ of kanamycin (Braun et al., 1997).

N-terminal amino acid sequencing

Crude L. monocytogenes surface proteins were extracted from the wild-type F4244 strain, as described (Santiago et al., 1999), and separated by SDS-PAGE (7.5% acrylamide). The 104 kDa bands were identified, excised, and eluted by electroelution (Bio-Rad, Hercules, CA). The process was repeated twice to eliminate contaminating proteins. The protein was transferred to a PVDF membrane (Millipore, Billerica, MA) for microsequencing (Prosize 492, Applied Biosystems, Foster City, CA) at the Purdue Protein Sequencing Laboratory. A section of the membrane was immunoprobed with the LAP-specific MAb-H7 (Pandiripally et al., 1999) to confirm the identity. Sequencing was repeated twice with separate sample preparations. A sequence homology search using the FASTF program ver 3.3t 07 (Pearson & Lipman, 1988) and BLASTP program of NCBI (Altschul et al., 1997) indicated LAP is an alcohol-acetaldehyde dehydrogenase (Aad) homologue.

Construction of a recombinant Escherichia coli expressing LAP

Genomic DNA from L. monocytogenes F4244 was extracted and the entire lap (aad) was amplified by PCR using E-LAP-F3 and E-LAP-R2 primers (Integrated DNA Technologies, Coralville, IA) (Table 1). PCR reaction contained 100 ng of template genomic DNA, 25 pmol of each primer and 10 mM of dNTPs and 1 μL of Taq polymerase (0.5 U μL⁻¹; Applied Biosystems). The PCR condition was as follows using a thermocycler

Table 1. Bacterial strains, plasmids, and primers used in this study

<table>
<thead>
<tr>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Listeria monocytogenes F4244</td>
<td>Our collection</td>
</tr>
<tr>
<td>L. monocytogenes A572</td>
<td>Pandiripally et al. (1999)</td>
</tr>
<tr>
<td>Escherichia coli DH5α-MCR F</td>
<td>Life Technology</td>
</tr>
<tr>
<td>E. coli BL21(DE3)</td>
<td>Novagen</td>
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<tr>
<td>E. coli pET32a</td>
<td>This study</td>
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<tr>
<td>E. coli pET32a-ELAP-2</td>
<td>This study</td>
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<tr>
<td>Plasmids</td>
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<tr>
<td>pGEMT-Easy</td>
<td>Promega</td>
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<tr>
<td>pET-32a</td>
<td>Novagen</td>
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<tr>
<td>pELAP-MIX</td>
<td>This study</td>
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<tr>
<td>pET32a-ELAP-2</td>
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<tr>
<td>Primers</td>
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<tr>
<td>Listeria adhesion protein (alcohol acetaldehyde dehydrogenase)</td>
<td>This study</td>
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<tr>
<td>E-LAP-F3 5'-GGGCCCAGCATTGGCAATTAAAGAAAAATGGC-3'</td>
<td>This study</td>
</tr>
<tr>
<td>E-LAP-R2 5'-CTCGAGAACACCTTTGTAAGGTTCAAGG</td>
<td>This study</td>
</tr>
</tbody>
</table>

Underlined sequence contains artificial enzyme digestion sites for SmaI and KpnI. *Site for NotI. †Site for XhoI.
(GeneAmp PCR System 9700, Applied Biosystems); hot start at 95°C for 5 min; 30 cycles with denaturation at 95°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 3.5 min; extra extension at 72°C for 10 min. The amplified DNA was resolved in 1% agarose gel, visualized by ethidium bromide staining and purified by using a gel extraction kit from Qiagen (Valencia, CA). The amplified DNA was cloned into pGEMT-Easy Vector (Promega), and designated pELAP-MIX. The plasmid, pELAP-MIX was digested with NotI and XhoI and inserted into expression vector pET-32a (Novagen, San Diego, CA), resulting in pET32a-ELAP-2. Finally, the construct, pET32a-ELAP-2 was transformed into the E. coli strain, BL21(DE3) (Novagen). Expression of LAP in E. coli was achieved by adding 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) solution into the exponential culture for 2 h and monitored by indirect ELISA and western blotting described below.

**Analysis of LAP expression in recombinant Escherichia coli by ELISA**

Indirect ELISA (Bhunia et al., 1991) was carried out with the whole and lysed bacterial cells to determine possible expression of LAP on the surface of the recombinant E. coli BL21(DE3) with pET32a-ELAP-2 and the parental strain BL21(DE3) with pET-32a, and L. monocytogenes F4244 (WT) and A572 (lap−). Five milliliters of each culture were centrifuged and washed, and cell pellets were resuspended into carbonate coating buffer, pH 9.6. One milliliter of each culture was lysed by sonication (five cycles of 5 min on ice) using a Branson Sonifier 150D (Niantic, CT) and centrifuged (16 000 × g, 10 min), and the supernatant (10 µL/well) was analyzed by SDS-PAGE.

For western blotting with L. monocytogenes WT and A572 (lap−), surface proteins were extracted as previously reported (Bhunia et al., 1991). Briefly, the cells were harvested from each 20 mL of culture and resuspended in 150 µL of sample solvent and incubated for 1 h at 37°C. The suspensions were centrifuged (16 000 × g for 10 min) and the supernatants (30 µL per well) were analyzed by SDS-PAGE (10% acrylamide). After transferring to Immobilon-P membranes (Millipore), proteins were probed with MAb-H7 (Pandiripally et al., 1999).

**Analysis of LAP expression in recombinant Escherichia coli by western blotting**

Western blotting was also performed to determine the expression of LAP in recombinant E. coli or the parental strain. Twenty milliliters of each culture were centrifuged and washed, and cell pellets were resuspended into 1 mL of 20 mM phosphate-buffered saline, pH 7.0 (PBS). Cell suspensions were sonicated (2 cycles of 1 min on ice) using a Sonifier 150D (Branson,) and centrifuged (16 000 × g, 10 min), and the supernatant (10 µL/well) was analyzed by SDS-PAGE.

**Adhesion analysis of recombinant Escherichia coli to Caco-2 cells**

To determine adhesion properties of recombinant E. coli, the Caco-2 cell line (HTB37) was used as described (Gaillard & Finlay, 1996). Listeria monocytogenes F4244, A572 (lap−), the recombinant E. coli strain, and a parental strain were added at about 10:1 ratio to Caco-2 monolayers and incubated for 1 h. Excess bacterial cells were removed by washing, Caco-2 monolayers were treated with Triton-X 100 (0.1%), and serially diluted. Bacterial adhesion was enumerated by plating as described previously (Pandiripally et al., 1999). To determine the effects of exogenous rLAP on the adhesion, Caco-2 monolayers were preexposed to purified rLAP (1 µg mL⁻¹; this concentration was found to be most effective as determined from our unpublished experiment) for 1 h at 37°C. Monolayers were washed with cell culture medium to remove excess rLAP and bacterial cells were added at about 10 : 1 ratio and adhesion assay was carried out as above.

**Purification of recombinant listeria adhesion protein (rLAP) and internalin B (rInlB) by nickel affinity column**

Recombinant E. coli pET32a-ELAP-2 was grown in LB (500 mL) at 37°C in a shaker incubator at 150 r.p.m. (New Brunswick Scientific). When optical density (OD₆₀₀) reached to 0.6, IPTG at 1 mM was added and grown for additional 3 h to induce LAP expression. The culture was centrifuged (6000 g, 10 min), the pellet was washed with PBS.
and held overnight at −80 °C. The pellet was next thawed in ice-water, sonicated as above and centrifuged (16 000 g, 10 min). The supernatant containing His-tagged recombinant LAP was purified using a Ni affinity chromatography according to manufacturer’s instructions (Novagen, San Diego, CA). The eluent was concentrated and washed using a Centricron concentrator (Millipore) with a 30-kDa molecular cut-off. The protein concentration was determined by the Bradford method (Bio-Rad). Similarly, His-tagged InlB was purified from recombinant E. coli strain BL21 (DE) with pET28b-1 using Ni affinity chromatography as previously described (Braun et al., 1997). Purity of LAP and the InlB was confirmed by western blotting using the LAP-specific MAb-H7 (Pandiripally et al., 1999) and InlB specific PA404 (Lathrop, 2005).

**Binding analysis of recombinant LAP with receptor Hsp60 in a surface plasmon resonant mirror biosensor**

Binding kinetics of purified rLAP with the eukaryotic receptor, Hsp60 was investigated in an IAsys sensor (Affinity Sensor, Cambridge, UK) according to the general procedure of Dmitriev et al. (2003). Briefly, 14.5 μg of purified human Hsp60 (Stressgen Biotechnologies, Victoria, British Columbia, Canada) was covalently coupled to the N-hydroxysuccinimide (NHS)/N-ethyl-N-(diethylaminopropyl) carbodiimide (EDC)-activated carboxylate surface of the cuvette as described previously (Lathrop et al., 2003). The association and dissociation phases, measured in arcsec, of serially diluted samples of rLAP (3.65–75 nM), anti-Hsp60 antibody (6.2550 nM), and rInlB (12.5–100 nM) were determined. Serially diluted test samples (50 μL) were loaded per well in duplicate at room temperature (22 °C); binding readings were acquired for 5 min. The data were compared with binding of anti-Hsp60 monoclonal antibody (Stressgen Biotechnologies, Victoria, BC). Binding parameters for protein interactions with Hsp60 was determined using Graphpad software (San Diego, CA). Observed rate constant values, $k_{on}$, were plotted against their respective ligand (anti-Hsp60 antibody and rLAP) concentrations; the slope of the line represents the association rate constant $k_a$ (M$^{-1}$ s$^{-1}$). The intercept of the line represents the dissociation rate constant $k_d$ (s$^{-1}$). The dissociation equilibrium constant $K_D$ is a measure of the binding strength. $K_D$ was calculated using the following formula: $K_D = k_d/k_{on}$.

**Statistical analyses**

Enzyme-linked immunosorbent assays were repeated on three separate plates with three replications per plate. Each adhesion experiment was repeated on three separate plates, with four replications per plate. Bacterial counts (CFU mL$^{-1}$) from adhesion assays, and ELISA absorbance values were analyzed using the Generalized Linear Model (GLM) procedure of SAS, and significant differences were determined according to Duncan’s test (SAS institute, Cary, NC, 1988). When appropriate pairwise studentized $t$-test was used to show differences at $P < 0.05$.

**Results and discussions**

**N-terminal sequence of LAP**

Western blotting of protein preparation from *Listeria monocytogenes* F4244 (WT) showed a strong reaction with anti-LAP MAb-H7 while the lap-negative mutant A572 did not show any reaction confirming the absence of LAP in this strain (Fig. 1). LAP bands from WT strain were collected from an unprobed membrane and micro-sequenced to identify the protein for further cloning of the lap gene. Fourteen amino acid residues (NH$_2$–Ala–Ile–Lys–Glu–Asn–Ala–Ala–Glu–Val–Leu–Glu–Val–Gln) were obtained and the sequence matched with *lmo1634* of the *L. monocytogenes* EGD genome sequence (Glaser et al., 2001) and designated LAP as an alcohol acetaldehyde dehydrogenase (aad) homologue. A BLAST homology search with the NCBI protein database showed 63–75% similarity with Aad found in six bacterial and a protozoan species (Table 2). A conserved-domain search revealed LAP (Aad) contains an iron-containing alcohol dehydrogenase domain at the N-terminus and an aldehyde dehydrogenase domain at the carboxyl end. The putative molecular mass of LAP in EGD was $M_r$ 94.69 kDa, which is close to the estimated $M_r$ of 104 kDa (Pandiripally et al., 1999).

*lmo1634* is the leading gene in an operon containing an unknown protein (*lmo1635*), an ATP-binding protein

![Fig. 1](https://academic.oup.com/femsle/article-abstract/256/2/324/632244)
(lmo1636) and a membrane protein (lmo1637) (Glaser et al., 2001). However, the presence of a putative promoter including a Shine Delgarno sequence upstream and a transcription termination site (ΔG = −22.0 kcal mol⁻¹) downstream of lmo1634 indicated this possibly could be monocistronically transcribed. The genes located upstream of aad are involved in tryptophan biosynthesis in opposite orientation (Glaser et al., 2001).

Expression of LAP in recombinant Escherichia coli

Western blotting revealed the expression of 104 kDa LAP in the recombinant E. coli (pET-ELAP-2) that reacted with MAb-H7 and absence in the parental E. coli strain (pET-32a) (Fig. 1). Protein preparation from L. monocytogenes F4244 strain (WT) and the purified recombinant LAP showed positive reaction with MAB-H7. Purified InlB also showed positive reaction with anti-InlB PAb404 (Fig. 1). Purified recombinant LAP and InlB were later used in binding kinetics assay in a surface plasmon resonance sensor.

In ELISA when whole cells were used, the recombinant E. coli strain pET-ELAP-2 had significantly higher (2.6-fold) reaction than the parental strain, suggesting surface expression of LAP in recombinant E. coli. Low levels of cross-reactivity in parental E. coli in ELISA are not surprising due to the presence of a LAP homologue in E. coli (Ho & Weiner, 2005). However, in western blot there is no reaction with protein preparation from parental E. coli suggesting antibody possibly reacts with a conformational epitope in LAP. L. monocytogenes WT had twofold higher expression than the recombinant E. coli. As expected the lap-mutant (A572) showed no reaction (Fig. 2a). We also analyzed total LAP expression by using mechanically (sonicated) lysed cells, and data showed that LAP expression in recombinant E. coli was equivalent to L. monocytogenes WT, and the values were about 6.6-fold higher than the parental E. coli strain. Taken together, these data suggest that a majority of LAP is intracellular and a small fraction is surface exposed as confirmed previously by our transmission electron microscopic study (Jaradat et al., 2003).

To verify ELISA data showing surface expression of LAP on recombinant E. coli cells, and not due to leakage of intracellular LAP from cells during ELISA experiment, we performed β-galactosidase (a 116 kDa cytoplasmic membrane bound enzyme) release assay as an indicator of cell lysis from whole cells and compared that with mechanically lysed cell preparations of parental and recombinant E. coli strains used in ELISA. In both strains, lysed cell preparations showed significantly higher (> 1.8-fold) β-galactosidase activity than whole cell preparations, indicating that bacteria in whole-cell preparations used in ELISA were mostly intact (Fig. 2b). Low levels of leakage from lysed cells during ELISA

| Table 2. Percent homology of alcohol acetaldehyde dehydrogenase (Aad) from Listeria monocytogenes with Aad homologues from other microorganisms |
|-----------------|-----------------|-------------|
| Microbes        | NCBI accession  | Homology (%)|
| Staphylococcus aureus | BAB93988       | 75          |
| Pasteurella multocida   | NP_246392      | 70          |
| Streptococcus agalactiae | AAN98961      | 69          |
| Clostridium acetobutylicum | NP_149199     | 63          |
| Escherichia coli          | AAC74323       | 63          |
| Entamoeba histolytica    | CAA54388       | 66          |

Fig. 2. (a) Indirect enzyme-linked immunosorbent assay (ELISA) analysis of surface and total Listeria adhesion protein (LAP) expression in Listeria monocytogenes and recombinant Escherichia coli. LAP expression for whole cells of recombinant E. coli pET-ELAP-2 whole cells was significantly (P < 0.05) higher than the parental strain pET-32a. Similarly, L. monocytogenes WT had significantly higher LAP expression than the mutant A572 strain. Lysed recombinant E. coli pET-ELAP-2 had significantly higher LAP than the parental strain. Furthermore, total LAP expression in pET-ELAP-2 appeared to be equivalent to L. monocytogenes WT. (b) Analysis of β-galactosidase (β-gal) release assay from whole cells and mechanically lysed E. coli cells used in ELISA. Lysed parental and recombinant cells had significantly higher activity than whole cells suggesting that there were no apparent lysis or leakage of β-gal from whole cells. If lyses occurred during ELISA experiment, β-gal activity would be similar for both whole cell and lysed cell preparations.
experiment may be considered as background reaction. If large-scale lysis had occurred during ELISA experiment, β-galactosidase activity in intact cells would have been equivalent or closer to values obtained from lysed cells.

To determine the level of LAP expression in recombinant E. coli, we monitored the expression of a highly expressed indicator membrane protein β-lactamase (Francisco et al., 1992) using a specific antibody in parallel with anti-LAP MAb-H7 in ELISA. Data showed that in parental E. coli, β-lactamase expression was 2.5-fold greater than LAP expression (0.8 ± 0.27 vs. 0.32 ± 0.02). However, in the recombinant E. coli, total LAP expression was 1.6-fold greater than β-lactamase (2.06 ± 0.41 vs. 1.28 ± 0.32), indicating possible overexpression of LAP in the recombinant strain.

**Adhesion characteristics of recombinant *Escherichia coli***

In the Caco-2 cell adhesion assay, the recombinant E. coli exhibited 1.04% adhesion compared to only 0.36% by the parental strain (P < 0.05). Similarly, *L. monocytogenes* WT showed 4.5% adhesion while the mutant A572 strain had only 0.22% adhesion (Fig. 3). When adhesion assay was performed with LAP-preexposed Caco-2 cells, there were about 2.8-fold reduction in adhesion for recombinant E. coli and 2.2-fold reduction for *L. monocytogenes* WT indicating that exogenous LAP occupied the binding sites on Caco-2 cell surface, thus reducing available binding sites for bacteria associated LAP. As expected exogenous LAP had no effect on parental E. coli or lap-negative *L. monocytogenes* A572 strain. These data confirm that LAP from *L. monocytogenes* can be expressed in E. coli and demonstrates its adhesion function using a standard Caco-2 cell-based adhesion assay. Similarly, Park et al. (2000) used a recombinant E. coli strain to demonstrate the functional adhesive property of *L. monocytogenes* p60 protein. Adhesion and invasion functions of InLA were also demonstrated in typically nonadhesive, noninvasive *Listeria innocua* (Gaillard et al., 1991) and *Lactococcus lactis* (Guimaraes et al., 2005).

Genetic and phenotypic analyses indicate that LAP is an alcohol acetaldehyde dehydrogenase (Aad) homologue involved in adhesion in *L. monocytogenes*. The *aad* (*lap*) possesses 63 to 75% sequence similarities with Aad from other bacteria and protozoan species (Table 2). In the protozoan, *Entamoeba histolytica*, the Aad enzyme (designated EhADH2; 97 kDa) has been recognized as a major adhesion factor that interacts with eukaryotic cells (Yang et al., 1994; Espinosa et al., 2001). EhADH2 is primarily located in the cytosol (Torian et al., 1990), similar to LAP from *L. monocytogenes* (Jaradat et al., 2003). In *E. histolytica*, this protein plays a critical role in adhesion to fibronectin in the host cells (Yang et al., 1994). Torian et al. (1990) indicated EhADH2 is present primarily in pathogenic strains and absent or present in small quantities on six nonpathogenic strains, suggesting the importance of this protein in pathogenesis.

Several precedents in which cytosolic enzymes serve as an adhesion factor in bacteria have been reported. A 41 kDa group A streptococcal plasmin-binding protein (Broder et al., 1991), identified as glyceraldehyde-3-phosphate dehydrogenase (GADPH) binds to fibronectin, lysozyme, myosin, and actin (Pancholi & Fischetti, 1992; Winram & Lottenberg, 1996) and may play a role in host–pathogen communication (Pancholi & Fischetti, 1993). Amidase and glucosaminidase from *Staphylococcus saprophyticus* was reported to possess autolytic activity and adhesive properties (Hell et al., 2003). A 102 kDa autolysin, expressed in *L. monocytogenes* and identified as an amidase, possesses the ability to hydrolyze peptidoglycan (McLaughlan & Foster, 1998). This autolytic amidase (ami) later demonstrated dual function as an adhesion protein (Milohanic et al., 2000; Milohanic et al., 2004). Ami-mutants showed significantly reduced binding in Caco-2 enterocytes, HepG2 hepatocytes, and SK-MEL 28 melanoma cells (Milohanic et al., 2001).

**Binding kinetics of rLAP to receptor Hsp60***

Surface plasmon resonance (SPR) allows real time monitoring of interaction between non-labeled biological molecules.
The successful immobilization of Hsp60 to carboxylate dual well cuvette was indicated by a typical binding curve (Pazos et al., 2005) (data not shown). A concentration-dependent increase in binding response (arcsec) was observed as anti-Hsp60 antibody (Fig. 4a) and rLAP (Fig. 4b) concentration was increased from 6.25 nM to 50 nM and 3.63 nM to 75 nM, respectively. The $k_a$ for rLAP was calculated to be $5.35 \times 10^6$ M$^{-1}$s$^{-1}$ and anti-Hsp60 was $2.15 \times 10^9$ M$^{-1}$s$^{-1}$. The $k_a$ indicates that both molecules exhibited similar binding to Hsp60 (Fig. 4b–d). InlB, another adhesion/invasion protein from L. monocytogenes used as a negative control in the range of 12.5–100 nM range exhibited a significantly lower binding ($2.9 \times 10^6$ M$^{-1}$s$^{-1}$) than LAP. Likewise, BSA (3–5 μM), also did not exhibit significant binding ($k_a = 0.789$ M$^{-1}$ s$^{-1}$). The $K_D$ for rLAP was calculated to be $1.68 \times 10^{-6}$ M and it was significantly lower than the $K_D$ value of InlB ($6.5 \times 10^{-4}$ M). The $K_D$ values obtained here fall within the range for biological molecules of $1 \times 10^{-4}$ to $1 \times 10^{-11}$ M (Pazos et al., 2004). Since the estimate of $k_d$ for anti-Hsp60 was negative, (i.e. negative intercept for $k_{on}$ values plotted against protein concentrations – Fig. 4b) the $K_D$ value for anti-Hsp60 was not determined. The binding response of wild-type LAP (104 kDa), purified from SDS-PAGE with Hsp60 protein, demonstrated strong binding when compared with a randomly selected protein (130 kDa) from the same strain (data not shown). These data strongly indicate that LAP binding is very specific to its cognate receptor Hsp60 exhibiting a high rate of association $c$. 1/4 that of anti-Hsp60 antibody. InlB which binds to its cognate Met receptor tyrosin kinase (Shen et al., 2000) had a negligible binding with Hsp60, again reiterating specific interaction of LAP with Hsp60.

Surface plasmon resonance has been widely used in recent years as a powerful analytical tool to study real-time molecular binding kinetics (Malmqvist, 1993; Rich & Myszko, 2000). Schaumburg et al. (2004) demonstrated strong binding kinetics of human plasminogen to enolase from L. monocytogenes using this technique. Binding kinetics of Yessotoxin, a diarrhea-causing toxin produced by microalgae, with immobilized phosphodiesterase has also been characterized by SPR (Pazos et al., 2004; Pazos et al., 2005). SPR has also been employed to detect point mutations in DNA (Momynaliev et al., 2003). Use of SPR in the field of pathogenesis is relatively new and holds great promise in unraveling mechanisms involved in the infectious process.

The adhesion and invasion of L. monocytogenes to intestinal cells is mediated by several proteins including InlA, InlB, Ami, Fbp, and LTA (Dussurget et al., 2004).
Identification and characterization of receptors for each of these proteins will clarify the L. monocytogenes pathogenic mechanism. InlA initiates infection through specific interaction with E-cadherin in intestinal cells (Lecuit et al., 2001). InlB binds to the C1q-R complement protein or a tyrosine kinase MET receptor (Shen et al., 2000). Involvement of LAP in pathogenesis, possibly during the intestinal phase of infection, has been previously demonstrated (Jaradat et al., 2003) and Hsp60 was identified as the mammalian LAP receptor (Wampler et al., 2004). The current study indicates LAP binding is rapid and very specific for the Hsp60 receptor.

In summary, LAP is identified as an Aad homologue and its adhesion function to eukaryotic cells is confirmed in a recombinant E. coli strain. SPR analysis indicated the interaction of LAP to its receptor, Hsp60, is very specific with binding kinetics equivalent to the anti-Hsp60 antibody.

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