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Serodiagnosis of Listeriosis Based upon Detection of Antibodies against Recombinant Truncated Forms of Listeriolysin O

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Amino-terminal fragments of listeriolysin O (LLO) of 240 and 411 residues (fragments LLO240 and LLO411, respectively) were expressed in *Escherichia coli* as fusion polypeptides with maltose-binding protein (MBP) with the aim of producing specific antigens for use in serological tests. In Western blots (immunoblots) with crude bacterial extracts of the fusion polypeptides, the reactivities of MBP-LLO240 and MBP-LLO411 with anti-LLO antibody (ALLO)- and anti-streptolysin O antibody (ASLO)-positive human sera were first compared with that of the entire LLO (LLO530) also fused to MBP (MBP-LLO530). Sixteen of 17 (94.1%) ALLO-positive samples reacting with MBP-LLO530 also reacted with MBP-LLO411, whereas this proportion dropped to 11 of 17 (64.7%) with MBP-LLO240. Alternatively, 18 of 19 (94.7%) ASLO-positive samples giving an interpretable result reacted with MBP-LLO530, whereas 1 of 19 (5.3%) of these samples reacted with MBP-LLO240 or MBP-LLO411. The fusion polypeptide MBP-LLO411 was purified by maltose affinity chromatography and was further evaluated as a diagnostic antigen in a Western blot assay. Twenty-one of 21 (100%) serum samples obtained from patients with listeriosis and found to be positive for ALLO by a reference dot blot test reacted with MBP-LLO411, whereas 1 of 20 (5%) ASLO-positive serum samples and 1 of 100 (1%) serum samples from healthy adults were reactive. Thus, a polypeptide limited to the 411 amino-terminal residues of LLO is a specific and sensitive antigen for the detection of ALLO.

Listeriosis is a sporadic and epidemic food-borne illness caused by the ubiquitous gram-positive organism *Listeria monocytogenes* (7, 11). Because of the high fatality rate among those with listeriosis, this disease is a problem of major concern to the public health community and food processing facilities in industrialized countries. Pregnant women and immunocompromised or aged individuals are primarily affected (11, 16). However, apparently normal, healthy individuals can also contract listeriosis. Infection of pregnant women may result in abortion, stillbirth, and neonatal meningitis or sepsis. Meningitis, meningoencephalitis, and bacteremia are the most common presentations in nonpregnant adults.

For diagnosing listeriosis, serological tests have long been unreliable tools, lacking sensitivity as well as specificity (11). They were negative with a large proportion of patients with culture-proven listeriosis, even in the absence of immunosuppression (15, 26). A high rate of false-positive reactions because of antigenic cross-reactions with components from other gram-positive organisms was observed (15, 23). Interest in the serodiagnosis of listeriosis has recently been renewed following the introduction of assays based on the detection of serum antibodies against listeriolysin O (LLO), a major virulence factor (3, 8) produced by all pathogenic strains of *L. monocytogenes* (13). Anti-LLO antibodies (ALLO) have been shown to be reliable indicators of listeric infections both in humans and in animals (2, 19, 20). Detection of ALLO in humans is particularly useful for diagnosing listeriosis of the central nervous system in patients with sterile blood and cerebrospinal fluid (10).

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LLO is a member of a family of pore-forming, thiol-activated cytolysins produced by some gram-positive organisms (27). Other prominent bacterial toxins belonging to this group include streptolysin O (SLO) from *Streptococcus pyogenes* and pneumolysin from *Streptococcus pneumoniae*. All of these toxins share immunological cross-reactivity. ALLO detection in humans can therefore yield false-positive results caused by the presence of antibodies against SLO, pneumolysin, and, potentially, any other thiol-activated cytolysin. Anti-SLO antibodies (ASLO) are a particular problem because they are found at substantial levels in a large proportion of the general population. This difficulty has been overcome in previous ALLO tests by absorbing ASLO prior to ALLO detection (2). However, ASLO absorption requires purified SLO, adds an additional step to the serological test, and is ineffective in removing antibodies against pneumolysin and other thiol-activated cytolysins produced by bacteria causing human infections. This may affect the sensitivity of ALLO assays by eliminating ALLO that are able to react against SLO. Moreover, the LLO antigen currently used in serological assays is extracted from *L. monocytogenes* cultures (2, 20), and this type of preparation exhibits considerable batch-to-batch variation. Finally, despite recent advances in this field (20), LLO is relatively difficult to purify (2, 12).

The genes for LLO, SLO, pneumolysin, and some other thiol-activated cytolysins have been isolated and sequenced (14, 17, 21, 28, 29). Comparison of the predicted amino acid sequences shows extensive homologies. However, although homologies are present along the whole sequence, they are substantially stronger toward the carboxy-terminal end. For example, the levels of residue identity between LLO and SLO are 43% in the carboxy-terminal halves but only 25% in the amino-terminal halves of the toxins (17, 21). The largest continuous stretch of identity shared by SH-activated hemolysins lies close

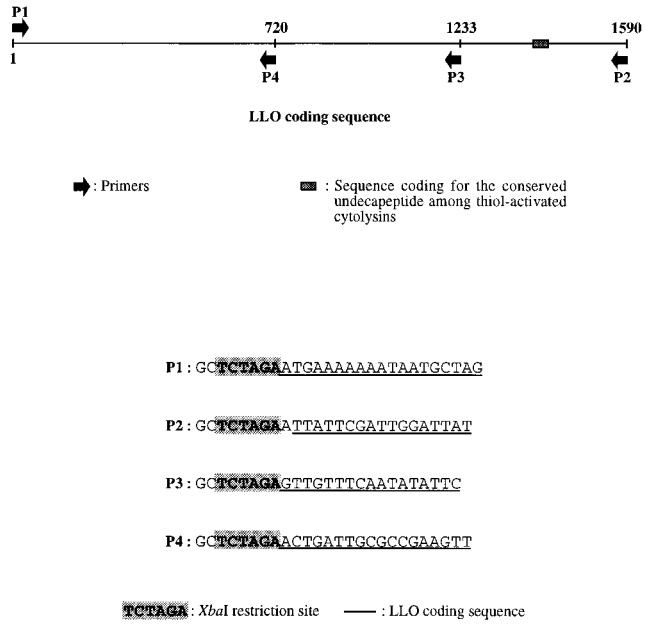

FIG. 1. Primers used for amplification of LLO-coding sequence fragments.

to their C-terminal ends. This conserved sequence consists of 11 amino acids and encompasses the only cysteine residue in SH-activated hemolysins. Mutational analysis studies have demonstrated that this undecapeptide is essential for the lytic activity of LLO (22).

We have assumed in the present work that amino-terminal fragments of LLO could contain LLO-specific epitopes. We describe the expression in *Escherichia coli* of recombinant truncated forms of LLO and the purification and evaluation of these fragments as antigens for the serodiagnosis of listeriosis. We show that a 411-residue amino-terminal fragment of LLO expressed as a fusion polypeptide with maltose-binding protein (MBP) is a specific and sensitive antigen for ALLO detection and renders previous ASLO adsorption from sera unnecessary.

MATERIALS AND METHODS

Human sera. The human serum samples tested in the present study included (i) 8 acute-phase (before day 7 of the disease) and 13 convalescent-phase (between days 14 and 28 of the disease) serum samples from 17 patients with culture-proven listeriosis (perinatal infection, 6 patients; meningitis and meningoencephalitis, 6 patients; bacteremia in nonpregnant adults, 3 patients; other, 2 patients), (ii) 20 ASLO-positive serum samples from 20 children aged 6 months to 14 years, and (iii) 100 serum samples from 100 healthy adults. ASLO-positive serum samples were selected from samples received for routine ASLO determination by the Laboratory of Microbiology of Necker-Enfants Malades Hospital. These samples exhibited ASLO titers ranging from 200 to 1,600 U, as determined with the ASL-kit (bioMérieux, Marcy-l'Etoile, France). Serum samples from patients with listeriosis and healthy adults, but not ASLO-positive serum samples (volumes of samples were insufficient), were assayed for ALLO by the dot blot test (2). All serum samples from patients with listeriosis were positive, with ALLO titers ranging from 100 to ≥800. All except one (ALLO titer = 400) of the serum samples from healthy adults were negative.

Construction of expression plasmids. DNA fragments corresponding to LLO sequences 1 to 1590 (entire LLO-coding sequence), 1 to 1233, and 1 to 720 were amplified by PCR by using the primers shown in Fig. 1 and chromosomal DNA from *L. monocytogenes* EGD-SmR (9) as template, which was prepared as described elsewhere (24). The primers, purchased from Société Bioprobe System (Montreuil-sous-Bois, France), were designed to contain 5′ *Xba*I restriction sites to facilitate cloning. PCR was performed by using 30 cycles of annealing (55°C, 1 min), extension (72°C, 1.5 min), and denaturation (95°C, 1 min). The amplified DNA fragments were purified with the Elutip-d kit (Schleicher & Schuell, Dassel, Germany). The DNA fragments were digested with *Xba*I (Boehringer, Mannheim, Germany) and were cloned into *Xba*I-digested, dephosphorylated (calf intestinal phosphatase; Boehringer) pMAL-p2 and pMAL-c2 plasmids (New England Biolabs, Inc., Beverly, Mass.) by standard procedures (25). *E. coli* MC1061 (5) cells were transformed by the ligation reactions. Transformants obtained on L agar plus ampicillin (100 mg/liter) were induced with isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, Mo.) (see below) and were examined for the production of fusion proteins of the expected sizes by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis. MC1061 derivative strains harboring either pMAL-p2 or pMAL-c2 without inserts were used as controls. Plasmid DNAs from transformants expressing fusion proteins of the expected sizes were digested with *Xba*I and were examined for inserts of the expected sizes. The junction areas of the constructs were verified by DNA sequencing (Sequenase kit no. 2; U.S. Biochemicals, Cleveland, Ohio.) *E. coli* TB1 was also used to express some fusion proteins; the constructs were introduced into TB1 competent cells by transformation (25).

Production of fusion proteins. Bacteria were grown at 30°C in L broth containing ampicillin (100 mg/liter) with shaking until the optical density (600 nm) reached 0.5. The cultures were then induced with IPTG (final concentration, 1 mM) and were harvested after 1 h of incubation at 30°C. Small-scale preparations were used for the screening of bacterial transformants and for the determination of optimal culture conditions. One-milliliter IPTG-induced cultures were centrifuged, and the resulting pellets were resuspended in SDS-PAGE sample buffer (100 mM Tris-HCl [pH 6.8], 2% SDS, 5% 2-mercaptoethanol, 10% sucrose, 0.01 bromophenol blue). One-liter IPTG-induced cultures were used for the large-scale production and purification of fusion proteins. Each culture was centrifuged, the resulting pellet was resuspended in lysis buffer (12.5 mM EDTA, 0.6 M sucrose, and 1 mg of lysozyme [Sigma] per ml in phosphate-buffered saline [PBS; pH 7.2]), and the mixture was incubated for 30 min at 37°C. The supernatant obtained after centrifugation of the bacterial lysate (periplasmic extract) was filtered and stored at −20°C. The pellet was resuspended in cold distilled water, and bacterial cells were lysed by freezing-thawing. The supernatant obtained after centrifugation (cytoplasmic extract) was filtered and stored at −20°C.

Maltose affinity chromatography. Maltose affinity chromatography was performed according to the manufacturer's protocol (New England Biolabs, Inc.). The cytoplasmic extract obtained as described above was loaded onto a maltose affinity column (Amylose Resin; New England Biolabs, Inc.). The column was washed with buffer A (50 mM Tris base, 15 mM NaCl [pH 7.5]) and was eluted with buffer A, to which 10 mM maltose (Sigma) was added. The fractions containing the fusion protein were detected by SDS-PAGE and/or Western blotting (immunoblotting) with rabbit anti-MBP (New England Biolabs, Inc.). These fractions were pooled and stored at −80°C. Protein levels were measured by the method of Bradford (4).

SDS-PAGE and Western blotting. SDS-PAGE was carried out as described by Laemmli (18) with 8% polyacrylamide minigels (Mini-PROTEAN II; Bio-Rad Laboratories, Richmond, Calif.). Loading concentrations were 10 to 15 μg per track. The proteins were transferred to nitrocellulose membranes (BA 85; Schleicher & Schuell) with a Mini TransBlot cell (Bio-Rad) in transfer buffer (25 mM Tris [pH 8.5], 0.2 M glycine, 20% [vol/vol] isopropanol). Transfer of proteins was confirmed by staining with ponceau S (Sigma). The membranes were blocked by incubation with washing buffer (0.15% Tween 20 in PBS [pH 7.2]) containing 5% skim milk for 1 h at room temperature. The membranes were then incubated for 45 min at room temperature with human serum (1/100 dilution), rabbit polyclonal anti-MBP antibody (1/5,000 dilution), or mouse monoclonal anti-LLO antibody (1/2,500 dilution), obtained by immunization of mice with purified LLO from *L. monocytogenes*. This was followed by incubation with an appropriate secondary antibody (1/1,000 dilution) for 45 min at room temperature. Secondary antibodies, all purchased from Organon Teknika (West Chester, Pa.), were peroxidase-conjugated goat anti-human, anti-rabbit, or anti-mouse immunoglobulin G. All sera and labeled antibody dilutions were prepared in washing buffer containing 5% skim milk. Antibody binding was revealed by adding 0.05% diaminobenzidine-tetrahydrochloride (Sigma) and 0.03% hydrogen peroxide.

RESULTS

Expression of recombinant LLO antigens in *E. coli*. DNA fragments corresponding to LLO amino acid sequences 1 to 530 (LLO530; the entire LLO sequence; predicted molecular size, 58.6 kDa), 1 to 411 (LLO411; predicted molecular size, 44.9 kDa), and 1 to 240 (LLO240; predicted molecular size, 26.4 kDa) were amplified by PCR and were inserted into vectors pMAL-p2 and pMAL-c2, and the resulting plasmids were expressed in *E. coli* MC1061 (Table 1). After induction of the bacteria with IPTG, lysates were found to contain fusion polypeptides with molecular masses consistent with the predicted masses of the translated sequences. The molecular masses of the major fusion products were about 110 kDa for p- and cMBP-LLO530 (where p and c represent periplasmic and cytoplasmic products, respectively), 95 kDa for p- and cMBP-LLO411, and 75 kDa for p- and cMBP-LLO240 (molecular

TABLE 1. Nomenclature for recombinant plasmids and fusion proteins

Expression plasmid	Insert[a]	Recombinant plasmid	Fusion protein[b]
pMAL-c2	1–1590	pYG101	cMBP-LLO530
pMAL-c2	1–1233	pYG102	cMBP-LLO411
pMAL-c2	1–720	pYG103	cMBP-LLO240
pMAL-p2	1–1590	pYG201	pMBP-LLO530
pMAL-p2	1–1233	pYG202	pMBP-LLO411
pMAL-p2	1–720	pYG203	pMBP-LLO240

[a] Numbers correspond to LLO coding sequence (1 is for the first base of the ATG start codon).
[b] LLO530, LLO411, and LLO240, LLO amino acid sequences 1 to 530 (entire LLO), 1 to 411, and 1 to 240, respectively.

mass of MBP, 42 kDa) (Fig. 2). A strong single band reactive with anti-MBP was found at about 45 kDa in all preparations, presumably corresponding to unfused MBP and/or fusion proteins cleaved at or near the junction site with MBP. Expression in pMAL-c2 gave 5- to 10-fold more fusion protein than expression in pMAL-p2 (data not shown). Fusion proteins from pMAL-c2 derivative constructs were therefore selected for subsequent studies. For unknown reasons, we also found that p- and cMBP-LLO240 were produced in smaller quantities than the fusion proteins containing LLO530 and LLO411.

Reactivities of ALLO- and ASLO-positive sera with LLO recombinant antigens. To determine whether cMBP-LLO411 and/or cMBP-LLO240 constituted more specific antigens than the entire LLO (cMBP-LLO530), we studied how ALLO- and ASLO-positive serum samples reacted with these different fusion proteins. Western blots were prepared from crude antigenic extracts and were probed with the sera (Fig. 3). The quantity of bacterial lysate loaded onto the gels was calibrated so that the complete form of each fusion protein gave a signal of similar strength with anti-MBP. Each of the serum samples, diluted 1/100, was tested simultaneously with the three fusion proteins. The response for a given fusion protein was categorized as positive (binding to the major form of the fusion protein), negative (no binding), or undetermined (binding to *E. coli* proteins). None of the serum samples recognized the MBP polypeptide at ~45 kDa. Thus, in all cases, the recognition of the fusion polypeptide resulted from the binding of antibodies to the LLO part and not the MBP part of the fusion.

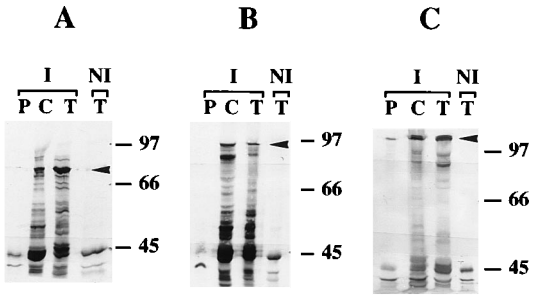

FIG. 2. Expression of fusion proteins cMBP-LLO240, cMBP-LLO411, and cMBP-LLO530 in *E. coli*. Cell extracts were prepared from bacterial cultures, induced or not with IPTG, and analyzed by Western blotting with rabbit anti-MBP antibody. Extracts were from *E. coli* MC1061 derivative strains expressing cMBP-LLO240 (A), cMBP-LLO411 (B), and cMBP-LLO530 (C). The molecular masses (in kilodaltons) are expressed to the right of each panel. I, induction with IPTG; NI, no induction with IPTG; T, total extracts; C, cytoplasmic extracts; P, periplasmic extracts. Arrowheads point to the complete forms of the fusion polypeptides. Note the strong reaction at ca. 45 kDa in all preparations.

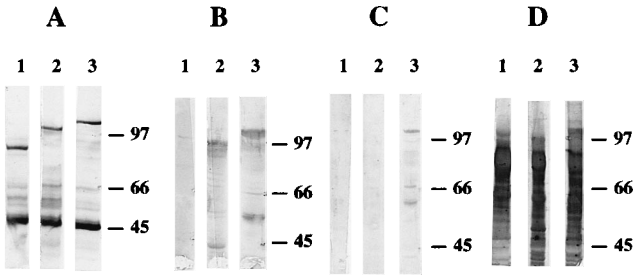

FIG. 3. Reactions of ALLO- and ASLO-positive sera against fusion proteins cMBP-LLO240, cMBP-LLO411, and cMBP-LLO530. Western blots were prepared by using bacterial lysates as sources of fusion proteins and were probed with human sera (diluted 1/100) or rabbit anti-MBP antibody (diluted 1/5,000). (A) Anti-MBP (note that the signal is similar for the complete form of each of the fusion proteins); (B) ALLO-positive serum sample from a patient with documented listeriosis; (C) ASLO-positive serum sample; (D) ASLO-positive serum sample reacting against *E. coli* proteins. Bacterial lysates were from *E. coli* MC1061 derivative strains expressing cMBP-LLO240 (lanes 1), cMBP-LLO411 (lanes 2), and cMBP-LLO530 (lanes 3). The molecular masses (in kilodaltons) are expressed to the right of each panel.

As shown in Table 2, 19 of 21 ALLO-positive and 19 of 20 ASLO-positive serum samples could be analyzed for their reactivities against the fusion proteins. Responses with the other samples were uninterpretable because of the presence of anti-*E. coli* antibodies. A total of 89.5% (17 of 19) of the ALLO-positive serum samples giving an interpretable result reacted with cMBP-LLO530. The proportion of interpretable responses that were positive was still 84.2% (16 of 19) with cMBP-LLO411, but the proportion dropped to 57.8% (11 of 19) with cMBP-LLO240. A total of 94.7% (18 of 19) of the ASLO-positive serum samples giving an interpretable result reacted with cMBP-LLO530, whereas 5.3% (1 of 19) of these samples reacted with cMBP-LLO411 or cMBP-LLO240.

Evaluation of MBP-LLO411 as a diagnostic antigen in a Western blot assay. The previous results showed that cMBP-LLO411 is a much more specific and only slightly less sensitive antigen than cMBP-LLO530 (the entire LLO sequence). Thus, we further evaluated the use of this recombinant protein for the detection of ALLO without preadsorption of serum samples with SLO.

The fusion protein cMBP-LLO411 expressed in *E. coli* TB1 was partially purified by maltose affinity chromatography and was analyzed by SDS-PAGE and the Western blot assay (Fig. 4). Two main bands, corresponding to fusion protein cMBP-LLO411 (95 kDa) and MBP (45 kDa), were detected. Each of these products represented >45% of the material stainable with Coomassie brilliant blue. Most of the extra bands revealed by Coomassie brilliant blue staining were recognized by the anti-MBP as well as the monoclonal anti-LLO antibodies and thus are probably degradation products or modified forms of the fusion protein. The final recovery of the fusion protein cMBP-LLO411 was about 1 to 2 mg/liter of culture. This amount was sufficient for about 1,000 individual tests.

The antigenic material obtained after maltose affinity chromatography was used in Western blots to test 21 serum samples collected from patients with culture-proven listeriosis and found to be positive for ALLO by the reference dot blot assay (2). Twenty ASLO-positive serum samples and 100 serum samples from healthy adults were studied as controls. Serum samples were tested at a dilution of 1/100 on the basis of preliminary experiments with serial dilutions of sera from test and control groups. The Western blot assay based upon purified cMBP-LLO411 compared well with the reference dot blot assay. A total of 21 of 21 samples from patients with listeriosis

TABLE 2. Reactivities of ALLO- and ASLO-positive sera with cMBP-LLO530, cMBP-LLO411, and cMBP-LLO240[a]

Serum sample	No. of serum samples with a response against the following:								
	cMBP-LLO530			cMBP-LLO411			cMBP-LLO240		
	Positive	Negative	Undetermined	Positive	Negative	Undetermined	Positive	Negative	Undetermined
ALLO positive (n = 21)	17	2	2	16	3	2	11	8	2
ASLO positive (n = 20)	18	1	1	1	18	1	1	18	1

[a] Sera were tested by Western blotting as described in the legend to Fig. 3. Responses were categorized as positive, negative, and undetermined (presence of anti-*E. coli* antibodies).

and only 1 of 100 samples from healthy adults reacted with the major form of cMBP-LLO411 (95 kDa). None of the samples, including those reacting with *E. coli* proteins (see above), recognized the MBP polypeptide at ~45 kDa. The serum sample in the negative control group that was positive by the Western blot assay was also positive by the dot blot assay (titer = 400). Thus, for the detection of ALLO, the results of the cMBP-LLO411 Western blot assay completely agreed with those of the dot blot assay. This was slightly better than our previous results obtained with a crude extract containing the fusion protein (Table 2), probably because purified cMBP-LLO411 was more accessible to the antibodies. Only 1 of 20 ASLO-positive serum samples was reactive, confirming the specificity of the cMBP-LLO411 antigen.

DISCUSSION

Two recombinant LLO proteins with C-terminal truncations have been produced. These antigens consisted of amino-terminal fragments of LLO of 240 (LLO240) and 411 (LLO411) residues and were only 25 and 31% identical to the corresponding fragments of SLO, respectively. Both fragments were devoid of the undecapeptide common to the SH-activated hemolysins, which starts at residue 483. These antigens were expressed in *E. coli* by using MBP as a fusion partner. MBP fusion proteins are relatively inexpensive to produce, can be purified in one step by maltose affinity chromatography, and can be easily standardized (6). They can be used as diagnostic antigens in a fused form because anti-MBP antibodies do not occur in the general population (1; this study). Alternatively, the MBP carrier can be cleaved with factor Xa because of a sequence coding for the recognition site of this protease just 5′ to the polylinker insertion sites of pMAL vectors.

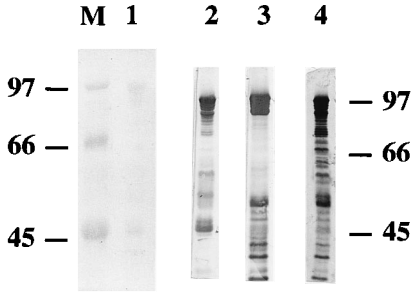

FIG. 4. Purification of cMBP-LLO411 by maltose affinity chromatography. Cytoplasmic extracts from *E. coli* cells expressing cMBP-LLO411 were loaded onto a maltose affinity column, and elution was achieved with 10 mM maltose. The fractions containing the eluted material were pooled and analyzed by SDS-PAGE (Coomassie brilliant blue-stained gel; lane 1) and Western blot assays with polyclonal rabbit anti-MBP (lane 2), monoclonal mouse anti-LLO (lane 3), or polyclonal rabbit anti-LLO (lane 4) antibodies. Lane M; molecular mass markers (in kilodaltons).

We demonstrated that LLO411 fused with MBP can be used for the specific detection of ALLO in human serum samples without the need for ASLO preadsorption. A Western blot assay based upon this antigen compared well with the reference ALLO dot blot assay. All serum samples collected from patients with culture-proven listeriosis and positive for ALLO by the dot blot test reacted with MBP-LLO411. MBP-LLO411 was also highly specific. Only 1 of the 20 ASLO-positive serum samples tested was reactive, and the only sample reactive among 100 serum samples from healthy adults was also positive by the ALLO dot blot test. The fusion protein LLO240 was as specific as LLO411 but was less sensitive. This indicates the presence of a number of important epitopes located between residues 241 and 410 of the LLO sequence.

Our results show that a 411-residue amino-terminal fragment of LLO is a suitable antigen for the development of sensitive and specific serological tests for ALLO. Convenient tests should ideally be solid-phase enzyme immunoassays. However, the production and the purification of polypeptide LLO411 need to be improved before developing an enzyme immunoassay based upon this antigen. We found that a large proportion of the fusion protein MBP-LLO411 produced in *E. coli* was cleaved at or close to the joint region of the fusion. This resulted in the production of large amounts of an MBP-like polypeptide that retained MBP's affinity for maltose. We were therefore unable to purify the fusion protein MBP-LLO411 to homogeneity by maltose affinity chromatography. A similar cleavage has been reported with other polypeptides fused with MBP; this is thought to be due to the sensitivity of the hybrid protein to host proteases (6). In our case, the presence of the LLO signal sequence in LLO411 might also constitute a target for proteases. Possible solutions to these problems are being investigated. They include the expression of an LLO polypeptide consisting of LLO411 without the signal sequence, the cleavage of the MBP carrier from the fusion protein cMBP-LLO411, or even the use of other expression systems.

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