iactA of *Listeria ivanovii*, Although Distantly Related to *Listeria monocytogenes actA*, Restores Actin Tail Formation in an *L. monocytogenes actA* Mutant

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A gene homologous to the *actA* gene of *Listeria monocytogenes* was cloned from *Listeria ivanovii* (strain CLIP257) by chromosome walking starting from the *ilo* gene that encodes the pore-forming toxin ivanolysin. The nucleotide sequence revealed that this gene, named *iactA*, encodes a protein of 1,044 amino acids (IactA) comprising a central region with seven highly conserved tandem proline-rich repeats of 47 amino acids. Although IactA and ActA share an overall similar structure, these two proteins are only distantly related. Like ActA, IactA migrates aberrantly on sodium dodecyl sulfate gels. When expressed in an *L. monocytogenes actA* deletion mutant strain, *iactA* restored actin polymerization.

Listeria ivanovii and Listeria monocytogenes are the only two pathogenic species of the genus Listeria. L. monocytogenes can infect humans and a wide variety of animal species. L. ivanovii is exclusively an animal pathogen (10, 33). Although taxonomically closely related, these two species differ in virulence (32). In the mouse, whatever the way of infection (intravenously, orally or peritoneally), the 50% lethal dose (LD_{50}) is higher for L. ivanovii than for L. monocytogenes. Nevertheless, the two species apparently use similar strategies to infect cells and tissues (34 and our unpublished data; for a review, see reference 52). The infection can be artificially divided into four successive steps: entry, escape from the phagocytic vacuole, intracellular multiplication, and direct cell-to-cell spread. This last step involves an actin-based intracellular movement which propels the bacterium in the cytosol and induces the formation of cellular protrusions each containing a bacterium at its tip. These protrusions are then endocytosed by the neighboring cell, resulting in the formation of a two-membrane vacuole. After lysis of this vacuole, a new cycle of intra- and intercellular spread can take place.

Genetic studies have been carried out mainly with L. monocytogenes. Several L. monocytogenes genes involved in the various steps of the infectious process are now known. Entry into epithelial cells is mediated by gene inlA, which encodes internalin (19). Entry into hepatocytes requires inlB, a gene encoding a protein which belongs to the internalin multigene family (16). Expression of another protein, named p60, is also important in the entry process (38). Escape from the phagocytic vacuole involves both listeriolysin O encoded by hly and the PI-PLC encoded by gene plcA (for references, see reference 52). Intra- and intercellular spread are mediated by actA for the actin polymerization process (for reviews, see references 11 and 12) and *plcB* for the lysis of the two-membrane vacuole (56). Interestingly, except for the inl genes and the iap gene coding for p60, all identified virulence genes are clustered in a chromosomal region which also encodes PrfA, a pleiotrocated upstream from *ilo* contains genes homologous to the *plcA* and *prfA* genes of *L. monocytogenes* (40). The region located downstream from *ilo* is homologous to the *L. monocytogenes* lecithinase operon including a putative *actA* homolog (25) which on the basis of hybridization signals was predicted to be only distantly related to *actA*. We report here the cloning and sequencing of the *actA* homolog of *L. ivanovii*. Its structure raises puzzling questions concerning the putative mode of action of the bacterial genes involved in actin-based motility. **MATERIALS AND METHODS**Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1 and Table 2, respectively. *Escherichia coli* strains were grown in Luria-Bertani broth, and *Listeria* strains were grown in brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.). Ampicillin

pic regulator of all known virulence genes but iap (59). We

have recently shown that this 15-kb locus is present in L.

ivanovii (25). It had previously been reported that the hly

homolog in L. ivanovii, ilo, encodes a closely related pore-

forming toxin named ivanolysin (27) and that the region lo-

coli strains were grown in Luria-Bertani broth, and *Listeria* strains were grown in brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.). Ampicillin was used at a final concentration of 25 mg/liter in broth and 100 mg/liter in agar plates. Erythromycin was used at a final concentration of 150 mg/liter for *E. coli* strains and at 8 mg/liter for *Listeria* strains. Nalidixic acid was used at a final concentration of 50 mg/liter in BHI agar plates.

Construction of the *actA* deletion mutant (LO28 $\Delta actA$). A 2.7-kb *Hind*III fragment containing the *actA* gene was isolated from plasmid pactA1 (36) and cloned into pUC18 (plasmid pactA14). An internal fragment of the *actA* gene was removed by restricting the vector with *Pml*I and *Bbs*I and isolating the vector fragment from an agarose gel by using the Geneclean kit (Bio 101, La Jolla, Calif.). The restricted ends were blunt-ended by T4 polymerase and religated. Several clones were sequenced and one, in which the T4 polymerase treatment had caused one nucleotide loss (leading to an in-frame deletion), was chosen for subcloning into *E. coli-Bacillus subtilis* shuttle vector pMK4 (plasmid pactA16.4). The modification of the *actA* gene leads to deletion of amino acids 35 to 576 (numbering as in reference 36) of the mature ActA protein and to insertion of a glutamine followed by a cysteine residue instead of the deleted residues.

actA deletion mutants were generated by using an LO28 (actA::Tn917lac) mutant (LUT12) by following a modified version of the method of Michel et al. (45). The strategy was to select for a double recombination event by which Tn917lac was exchanged with the in-frame truncated actA carried by plasmid pactA16.4. Since transposon-insertion into the actA gene causes a polar effect on the expression of the downstream lecithinase gene, restoration of lecithinase transcription owing to double recombination results in a lecithinase-positive phenotype that can easily be selected for on egg yolk agar plates. LUT12 was transformed with pactA16.4 by electroporation. One colony was picked, and a 5-ml overnight culture containing chloramphenicol (7 μ g/ml) was grown at 37°C with aeration. After 24 h, this culture was diluted 1:1,000 into 5 ml of fresh BHI

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Strain	Genotype or relevant properties	Collection name	Source or reference
E. coli			
MC1061	F^- araD139 Δ (ara-leu)7697 Δ lacX74 galU galK hsr hsm ⁺ strA		9
DH5aF'	F'/endA1 hsdR17 ($r_{\rm K} = m_{\rm K}^+$) supE44 thi-1 recA1 gyrA (Nal ^r) relA1 Δ (lacZYA-argF) ₁₁₁₆₉ (Φ 80lacZ Δ M15)		28 Gibco/BRL
S17.1	RP4-2 (Tc::Mu) (Km::Tn7), Tp ^r , Sm ^r Spc ^r thi pro hsdR hsdM ⁺ RecA		53
$DH5\alpha F'(pP1)$		BUG942	16
L. ivanovii			
ATCC19119 ^T		BUG496	J. Rocourt, Institut Pasteur
CLIP257		BUG497	J. Rocourt, Institut Pasteur
SLCC4121		BUG598	J. Rocourt, Institut Pasteur
subsp. londoniensis SLCC4054		BUG1188	5
L. monocytogenes			
LO28 (serotype 1/2c)		Bof 343	57
LO28 <i>dactA</i>		BUG875	This study
LO28 $\Delta actA(pIV11)$		BUG1121	This study

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with 7 µg of chloramphenicol per ml; this procedure was repeated six times. After 1 week, 100 µl each of a 10^{-4} and a 10^{-3} dilution was plated on egg yolk agar plates. *actA* deletion mutants showing egg yolk opacity were picked. They occurred with a frequency of about 1 in 3,000. The plasmid was cured from these strains by growing the bacteria in liquid culture at 42°C without antibiotics. After 24 h, this culture was diluted 1:1,000 into 5 ml of fresh BHI; this procedure was repeated three times. Then, 100 µl of a 10^{-4} dilution was plated, and colonies that had lost erythromycin resistance (indicating loss of Tn917lac) and chloramphenicol resistance (indicating loss of pMK4) were selected by replica plating. They occurred at a frequency of a bout 1 in 70 in three strains and with a frequency of 1 in 18 in one strain.

The LD_{50} was determined as described previously (23) with C3H mice. The LD_{50} (3.6 × 10⁷) of LO28 $\Delta act4$ (BUG875) was higher than that of LO28 (2.25 × 10⁴) and lower than that of LO28 *act4*::Tn917lac (LUT12) ($LD_{50} > 2.5 \times 10^8$). In accordance with previous results (8), this result indicates that inactivation of the *act4* gene leads to a strongly attenuated phenotype with an increase in LD_{50} by almost 3 orders of magnitude compared to the LD_{50} of the wild-type strain. The polar effect of the *act4* mutation on lecithinase expression in the LO28 *act4*::Tn917lac mutant LUT12 leads to a further increase in LD_{50} of less than 1 order of magnitude.

Construction of plasmid pIV11. Plasmid pIV11 was constructed by inserting the *SalI-SphI* fragment, carrying *iactA*, in plasmid pP1, which is a pAT18 derivative carrying a strong constitutive promoter inserted at the *Eco*RI site (16).

Transfer of plasmids. Plasmids were introduced into *E. coli* strains by transformation after CaCl₂ treatment (50) and into *Listeria* strains by conjugation as previously described (55), and transconjugants were selected on BHI agar containing nalidixic acid and other appropriate antibiotics.

Mammalian cell culture, infection, and double fluorescence labeling of F actin and bacteria. Vero cells (ATCC CCL 81) were cultured in Dulbecco's modified Eagle's medium containing glucose at 4.5 g/liter (Techgen Laboratories, Les Ulis, France), supplemented with 2 mM L-glutamine (Gibco, Cergy Pontoise, France) and 10% fetal calf serum (Boehringer, Mannheim, Germany). PtK2 cells (ATCC CCL56) were cultured in Eagle's minimal essential medium with nonessential amino acid, sodium pyruvate, and Earle's balanced salt solution (90%) supplemented with fetal calf serum (10%) and 2 mM L-glutamine.

Immunofluorescence of cells infected with bacteria was performed as described previously (37). Actin was revealed by using fluorescein isothiocyanatephalloidin. Bacteria were labeled with an antiserum raised against heat-killed *L. monocytogenes* at a dilution of 1/750 and with rhodamine-coupled goat antirabbit immunoglobulin G (Biosys, Compiegne, France). Preparations were observed with an Optiphot-2 epifluorescence microscope (Nikon).

Antiserum against heat-killed *L. monocytogenes.* LO28 was grown at an optical density at 600 nm of 0.6. Bacteria were centrifuged and then washed with phosphate buffer (pH 7.4), and the concentration was adjusted to 10^9 bacteria per ml. The bacterial suspension was heated at 60° C for 1 h. After verification that bacteria were killed, 1 ml of the suspension was emulsified with incomplete Freund's adjuvant and injected intradorsally and intradermically into a rabbit. Three and eight weeks after the first immunization, the rabbit was boosted with the same antigen in the same way. After 12 weeks, a third boost without adjuvant was given into footpads and intravenously. Rabbits were bled 10 days thereafter. This antiserum recognizes *L. monocytogenes* but neither *L. ivanovii* nor *L. innocua*.

DNA techniques. Standard recombinant DNA techniques were used for cleavage of DNA with restriction endonucleases (Boehringer, Mannheim, Germany, and Appligene, Illkirch, France), dephosphorylation of DNA ends with heat-killed phosphatase (Epicentre Technologies, Madison, Wis.), and ligation of DNA fragments with T4 DNA ligase (Amersham, Les Ulis, France) (50). Chromosomal DNA from *L. ivanovii* was prepared as described previously (44). Probes for Southern blots were prepared by enzymatic digestion, purified from agarose gels with the Geneclean kit and labeled by using the multiprime system of Amersham. Southern hybridizations were done with the rapid hybridization system (Amersham) on Hybond N nylon membranes (Amersham). *Taq* polymerase was obtained from Amersham.

A probe internal to the *ilo* gene was generated by PCR using the two oligonucleotide primers 5'GCGTATACAACTAATTTCTTG3' and 5'GGATTATC TACAGTATCACTA3' (positions 1153 to 1173 and 1497 to 1517, respectively [27]). The *iactA* gene of four different *L. ivanovii* strains was analyzed by PCR using the two oligonucleotide primers 5'GGAATCTTCGGTAAGTGAACCT AG3' and 5'CCTAAGTCCCCCCTCAATAAG3' (positions 1524 to 1547 and 2607 to 2627; see Fig. 3). Primers were purchased from GenSet (Paris, France).

Nucleotide sequencing. Plasmids used for sequence determination were purified with the Qiagen kit (Qiagen, Inc.). Sequences were determined on both strands of the DNA using the dideoxynucleotide chain termination method (51) with [³⁵S]dATP (600 Ci/mmol), the T7 sequencing kit from Pharmacia, and the universal primer or oligonucleotide primers derived from the sequence. To sequence inserts of plasmids pIV5 and pIV6, unidirectional deletions were generated with exonuclease III/S1 nuclease and the double-stranded nested deletion kit from Pharmacia and appropriately chosen subclones were used.

TABLE 2. Plasmids

Plasmid	Host (collection name)	Marker	Relevant properties ^a
pIV1	MC1061 (BUG937)	Amp	pUC18 derivative carrying part of <i>ilo</i> and <i>impl</i>
pIV2	MC1061 (BUG960)	Amp	pBR322 derivative carrying part of <i>impl</i> and <i>iactA</i>
pIV3	MC1061 (BUG962)	Amp	pBR322 derivative carrying the end of <i>impl</i> , <i>iactA</i> , and part of <i>iplcB</i>
pIV5	DH5aF' (BUG973)	Amp	pBluescript SK ⁺ derivative carrying a fragment internal to <i>iactA</i>
pIV6	DH5aF' (BUG974)	Amp	pBluescript SK ⁺ derivative carrying a fragment internal to <i>iactA</i>
pIV10	MC1061 (BUG978)	Amp	pUC18 derivative carrying a fragment internal to <i>iactA</i>
pIV11	MC1061 (BUG1118)	Ery	pP1 derivative carrying <i>iactA</i> under the control of a strong constitutive promoter

^a All plasmids are presented in Fig. 2.



BUG 598

BUG 1188

FIG. 1. PtK2 cells infected by four different *L. ivanovii* strains and stained with fluorescein isothiocyanate-phalloidin.

Computer sequence analysis. Sequences were analyzed either on a Macintosh with Gene Jockey (Biosoft) and DNA Strider (43) or on the mainframe computer run by the Service d'Informatique Scientifique-Institut Pasteur with the University of Wisconsin Genetics Computer Group package (version 7.1) (13) and Statistical Analysis of Protein Sequence (7). The Blast algorithm (1) was used to search the nonredundant protein and nucleic acid database compilations of the National Center for Biotechnology information (National Institutes of Health, Bethesda, Md.).

Nucleotide sequence accession number. The sequence depicted in Fig. 3 was deposited in GenBank under the accession number U19O35.

Protein analysis. Bacterial surface proteins were extracted with sodium dodecyl sulfate (SDS) as described previously (36) and were separated by SDSpolyacrylamide gel electrophoresis (SDS-PAGE) (39) in 6% acrylamide gels. For immunoblots, proteins were transferred onto nitrocellulose according to standard procedures (29), using a semi-dry transfer apparatus at 0.8 mA/cm² for 1 h at room temperature. Transferred proteins were detected with affinity-purified anti-ActA antibodies raised against a 21-amino-acid peptide of the repeat region of ActA. The antiserum was obtained as previously described (37). The antibodies were affinity purified over ECH Sepharose 4B (Pharmacia, France) coupled to the peptide according to the manufacturer's instructions. Anti-peptide antibodies were eluted with 0.1 M ethanolamine (pH 11.5), dialyzed against phosphate buffer (pH 7.4), and stored at -20° C in 50% glycerol-phosphate-buffered saline. The antibodies were used at 70 ng/ml and detected with anti-rabbit horseradish peroxidase-conjugated antibody (Biosys, Compiègne, France) and enhanced chemiluminescence (Amersham).

RESULTS

Cloning of the *iactA* **gene of** *L. ivanovii. L. ivanovii* is able to induce actin polymerization, move intracellularly, and spread from cell to cell, as shown by electron microscopy or fluores-

cence labeling with fluorescein isothiocyanate-phalloidin on permeabilized L. ivanovii-infected cells (34) (Fig. 1). In L. monocytogenes, the gene responsible for this intra- and intercellular movement is actA (15, 36). The recent discovery that the whole virulence gene cluster of L. monocytogenes, including actA, is also present in L. ivanovii (25), urged us to clone the L. ivanovii gene homologous to actA. We used a chromosomewalking strategy. We first cloned the chromosomal DNA region of L. ivanovii (strain CLIP257) located downstream from ilo, the gene homologous to hly of L. monocytogenes. A DNA fragment internal to ilo was amplified and used as a probe to clone a 2.3-kb HincII chromosomal fragment in pUC18, giving rise to plasmid pIV1. Partial sequence determination indicated that pIV1 contained the 3' end of *ilo* and the 5' end of a gene homologous to L. monocytogenes mpl, which we named impl. A 0.5-kb EcoRI fragment isolated from pIV1 was used as a probe to clone into pBR322 a 2-kb HindIII fragment (plasmid pIV2). Partial sequence determination indicated that it contained most of *impl* and the beginning of the L. ivanovii gene homologous to actA, which we named iactA (see below). Using the 0.35-kb EcoRI fragment of pIV2 as a probe, we cloned a 4.4-kb SphI fragment (pIV3). Sequence determination showed that pIV3 contained the end of *impl*, the whole *iactA* gene, and part of *iplcB*, the *L. ivanovii* gene homologous to *plcB* (see below and Fig. 2).

Nucleotide sequence of the *iactA* gene. The physical map and the genetic organization of the chromosomal region located downstream from *ilo* are depicted in Fig. 2. The nucleotide sequence of the 4,084 bp located between the *Sal*I and *Sph*I sites present on pIV3 was determined on both strands of the DNA (Fig. 3).

The first 99 nucleotides correspond to the 3' end of impl, which encodes a protein highly homologous to the metalloprotease of L. monocytogenes (83% similarity and 74% identity in a 30-amino-acid overlap). No palindromic sequence which could act as a transcription termination signal was found downstream from *impl*. The 3,240-bp open reading frame present 162 bp downstream from the stop codon of *impl* and in the same orientation encodes a protein homologous to ActA of L. monocytogenes and, as previously mentioned, was named iactA (see below). At 42 bp downstream from *iactA*, the sequence revealed the presence of a 531-bp open reading frame encoding a 177-amino-acid polypeptide highly similar to the lecithinase of L. monocytogenes (66% identity and 79% similarity on a 139-amino-acid overlap) and, as previously mentioned, named *iplcB*. Since no stop codon was detected, the sequence of *iplcB* presented here is only partial.

iactA encodes a protein similar but distantly related to the ActA protein of *L. monocytogenes.* The *iactA* gene encodes a protein of 1,080 amino acids (Fig. 4). The hydrophobicity profile indicates that the N- and C-terminal extreme parts of the



FIG. 2. Physical and genetic map of the *iactA* gene region of *L. ivanovii* CLIP257 (BUG497). Genes are represented by arrows under the restriction map. The inserts present in the plasmids used to sequence *iactA* are shown above the restriction map.

FIG. 3. Nucleotide sequence of the *SalI/SphI* fragment containing *iactA*. The protein sequence deduced from the nucleotide sequence is given in the single-letter code for the end of *impl*, the entire *iactA* gene, and part of *iplcB*. The signal sequence of IactA is underlined. The restriction sites *SalI* and *SphI* are indicated. Asterisks indicate stop codons. Numbers on the left indicate nucleotide coordinates.

 MACTAGATAGATTITIACIGGGAATGATGGCAGTITITIATTIGCAAGGATGTATCITIG K L D R F L L R A M R A V. C F T A S C I L GITTIACICGGTGATGTATTIGGAAGGATGAGCAGGCAGAAGGAAGTAGAGAA S N L E S D E O G E G E O A E G K V K E GUNAMACAGTICTIGGGCATGACAGTATAGCAGGCAGACGGAAGTAGAGAA S N L E S D E O G E G E O A E G K V K E GUNAMACAGTICTIGGGCATGACAATTATGTGGGAAGTAGACGAA S N S F G H G A I S E A C A R D I O E CCGGGAAAAAGGGTGAGCTAAAAGTAGCAGACGATGTAGCAGGTAACTAGGCAGGT L G K T G E A K S A N V P D S N T T P D AGGGATHAGTAAGGAGGAAGACGGAGAGTAAAAGTATGCGCGGATACTAATTAAAGGAGGAAGTAGACGAA F S V R K R P K R C V E K V L K G K R N R T F S V R K R F K R C V E K V L K G K R N AMACTAGATGAGAGAGAAAAGGGAGAGTAAAAGTAGCAGAAGTAAAGGAGAAATTAAAGGAAGG	241	CTGAATTGTATACTGTTCTTACGCAGGTATTAGAGAATGGCGAGGAGGAGGAGTAAAAAAGTG MA.R.R.S.K.K.V
 361 <u>JETERATEGENETISTER NUTTEGENETISTER CARCENTER CARCENT</u>	301	AAACTAGATAGATTTTTACGGCAGCAATGATGGCAGGTTGGTT
 121 TCTAACCTTGAATGAACAGAGAGAGAGAGAGAGGAGAAGGAGAAAGGAGAAAGTAGAAGA	361	GTTAATCCTGGTGTGATATTTGCAAGTAATGAGAAT <u>V.N.P.G.V.L.F.A</u> SNSTVSTSSNEN
 481 GENGLANCKGTECTGGGENGGGENGGENGGENGGENGGENGGENGGENGGENG	421	TCTAACCTTGAATCAGATGAACAGGGAGAAGGTAGGCAGGGAAGGGAAAGTAGAAGA SNLESDEQGEGEQAEGKVEE
 541 CTCGGMAMACGGTGAAGCTAAAAGTGGCAAATGTCCCGGATTCAATGTCACCCCCGAT 601 ACCGGATAAGTAATGACCGGCAGAGGATTTGGGAAAGCTAATGTAAGGAAT 601 CCGTCTGTGGGAAAGGATTAAACGCTGGGTGGGAAGGCTAAAGTAATAAAAGGTAAGGGAAT 601 P.S.V.R.K.R.P.K.R.C.V.E.K.V.L.K.G.K.R.N. 701 CCTAAGAACTGTGGCTGCTGCTGGTGGGATGGGAATGGGACTAGGAGGGGCTTGAAGGAGGGGCTGCACCGACGATAGGAAAGCAAGGAGGGGCTTGGGACGAGGGAGG	481	GGAAGAAACAGTCCTGGGCATGGAGCAATTAGTGAGGCGTGCGCCACGCGATATTCAAGAA G R N S P G H G A I S E A C A R D I Q E
 400 AGCGANTRAGTAAAGAACCAGAACAGAATATTGGAGAAACCGTAACTAAGTCTAAGTCTAAGCAA S G L S K E P G Q N I G G E T V T K S K P 601 COGTOTOGGAAAGAGATAGATTAAAACTACTAAGAAAAACTATTATAAGACAAT P S V R K R P K R C V E K V L K G K R N 721 MATCATCAAGGAAGAAACTAAAACTAACTAAGAAAATCAATTAGAGAAGTTCTAGAA K S S K E E T K T T K K K O E S S K L Q 731 OCTAAGAACTGGTGCCTACTCACTCAATAGCAATCAATCAA	541	CTCGGAAAAACGGGTGAAGCTAAAAGTGCAAATGTCCCGGATTCAATGACTACGCCAGAT L G K T G E A K S A N V P D S M T T P D
 661 CCGTCTGTCCCAMAACGATTTAAACGCTGCGTTGAGAAAGTATTAAAAGGTAAGAGGAAT PSVRKRFKRCCAMACGAMACGTAMACGCAGAAAGTATTAAAAGGTAAGAGGGAAT KSSKEETKTT KKKVQESSKACGATGCTGCCATAGCAAAGAAAAGCAAGCATTGAGGAGGCGCTTCAA PKSKRCTGGCTGATGGCGAAGGCGAAAGGAAAAGCAAGCGATGGGAGGGGAGGGA	601	AGCGGATTAAGTAAAGAACCAGGACAGAATATTGGAGAAACCGTAACTAAGTCTAAGCCA S G L S K E P G Q N I G E T V T K S K P
721AMATCATCAMAGGAGAMACTACTAACAAAAAGCAAATTAGCAAGCATCTAGCAACGACTTACAA K K S S K K E E T K K T T K K K Q E S S K L Q781CCTAMAGMACTGGCCCTCAATACCAATAGCAAATCAATTATTGGAGAGCCTTCAA P K E L V P H P S I A N Q S F W R R L S841GACAMATAMACCACTAGCAAACGAATGACAATCAATTGGAGAGCACACAACGAT D K I K P V V T S N D D N D S R I D S D901GATCGGATCAGTGGTAACGAAGCAATGACAAATGAACGATTGCAACGAACG	661	CCGTCTGTGCGAAAACGATTTAAAACGCTGCGTTGAGAAAGTATTAAAAGGTAAGAGGAAT PSVRKRFKRCVEKVLKGKRN
 CCTAMAGAACTTGTGCCTCATCCATCAATAGCAATCAATCATTTGGAGGAGGCCTTCCAT P K E L V P H P S I A N Q S F W R R L S GACAMATAAAGCCAGTAGTTACAAGCAACGATGATAACGATTGTGAGGATCGACTCAGAT D K I K P V V T S N D D N D S R I D S D GAATGGGATGATGGTGATGGTGAAGGCGAAAGAAAAGTAGGAGGAAGGA	721	AAATCATCAAAGGAAGAAACTAACTACTAAGAAAAAAGCAAGAATCTAGCAAGCTTCAAKKSSSKEETTEKKSSSKEETTEKKSSSKEEQ
841GACAMATAMAGECAGTAGTACAAGGACAACGATGATAACGATTCTAGGATCGACTCAGAT D K I K P V V T S N D D N D S R I D S D901GAATGGGATGATGGTGAAGGAGGGAAAGAAAAGTAAGGAGAGGAGGAGGGG	781	CCTAAAGAACTTGTGCCTCATCCATCAATAGCAAATCAATC
901 GAATGGGATGATGGTGAAGAGGGCGAAAGAAAAGTAGAGGAGGGAAGGAA	841	GACAAAATAAAGCCAGTAGTTACAAGCAACGATGATAACGATTCTAGGATCGACTCAGAT D K I K P V V T S N D D N D S R I D S D
961 AAGCGGAGGAAGAAAAAACAATCTCGGGGAAGGGAAATTAGTGAAGCGAGAGATAGG K A E E E K N N L G Q E E I S E A R D R 1021 GATCTCCAAGAACTCGAAGAAAGTAGCGGAAAGTTAAAAGCAATAGCGAGGGTTGCTGC D L Q E L E K M G K V K N A N V T A L L 1081 GCTATGCTGGATAGCAGGGCGGAAAGTAAGCAAGGAAATTATAAGGGAACTTTAAAT A M L D S R A G K V A R Q D I K E T L N 1141 GATGAGGTTCCAGCGTGCGTTTCACCCCCAAGGCGAGTATTAAAGAAAATATTAAGCGAC D E V P A V L F H P K R S I K E I L S D 1201 GAGCAAAATAGAGTACCATGAATAGTAGTAAGAAAAAGAAAAAGCATTGAA E Q N R V P M N S G K I K N R R K A I E 1261 GGATCTGATATGGAAGACTCTGATATGGAAGATGCTGATACAGAAAAAGCAATGGAA H G A T G P L R T M N P S T S E E T S E 1381 AAAATCCATCTAATGAGAATAATGAGAACAACTACGGAAGAAAAGCATCGGAA H G A T G P L R T M N P S T S E E T S E 1381 AAAATCCATCTAATGAGAATAATGAGGAAATCCAACAAGAAAAAGCATCGGAA H G A T G P L R T M N P S T S E E T S E 1381 AAAATCCAATCTAAGAATAATGAGGAAATCCTAGCAATCAAAGTCCGGAAA H G A T G P L R I A L P E P P G L L G 1501 TTCAATCTAACGGAAGAATAATGAGGAATTCTTCGGTAAGTGCAGTTGGTTTTATTA K I Q S N E N N E E S S V S E P S S F N L 1561 TCAATCTGACAGGAAGAGGCTTGCGCGCAACTGCGGAATGCGGCAAGGGATTACCGCTAAGTGTTACCCCA S S P P T E E E L A A M G I T L S V T P 1621 TCAATTGCAAAATAATGAATGGACTTGCGCCACAGGGAATGCGGCATAGGGCATAGCGCTAAGTGTTACCCCA S V E E E S S L H L P K E D A P Q S L T 1661 GCTATACGGCCAATCGAAGGAGGCTTGCCGCCACGGAAGAGGAGGCTTGCGCCCTCAGGTGCATTCAAGA S V E E E S S L H L P K E D A P Q S L T 1661 GATGCGCCTCAGTGGTAAGCGATTCCACCTAATGCGATAGGCCAATGCGACATCCAGGTGCGTTAACA S V E E E S S L H L P K E D A P Q S L T 1661 GATGCGCCTCAGTGGTAAGGAATCCACGCTAAGGCCAATGCCACCTCAGTGCGTTAACA S V E E E S S L H L P K E D A P Q S L T 1661 GATGCGCCTCAGTGGTAAGGCAATCCACGCTGCGCCACTCAGTGGATTACCG F P S P P T E E E L A A M D M K Q S I A P T V E G E S S L R P S R E 1861 GATGCGCCCTCAGTGGTTAACGCCTAATGCCCACCTCAGTCGAAGGAATGCAAGCATACCACATCCATC	901	GAATGGGATGATGGTGAAGAGGGGGAAAGAGGAAGGAAG
 1021 GATCTCCAAGAACTCGAAAAAAATGGGGAAAGTTAAAAATGCCAATGTAACGGCTTTGCTG D L Q E L E K M G K V K N A N V T A L L 1081 GCTATGCTCGATACCAGACCCGAAAAGTAGCAAGGCAAG	961	AAAGCGGAGGAAGAAAAAAAACAATCTCGGGCAAGAGGAAATTAGTGAAGCGAGGAGGATAGG KAEEKNNLGQEEISEARDR
1081CCTATECTCGATAGCAGAGCCGGAAAAGTAGCAAGGCAAG	1021	GATCTCCAAGAACTCGAAAAATGGGGAAAGTTAAAAATGCCAATGTAACGGCTTTGCTG D L Q E L E K M G K V K N A N V T A L L
1141 GATGAGGTTCCAGCGGTGCTCTTCACCCCAAAGGCAGTATTAAAGAATATTAAGGCAC D E V P A V L F H P K R S I K E I L S D 1201 GAGCAAAATAGAGTACCAATGAATAGTGGTAAGAAAAGAATAGAAAAAGCAATTGAA E Q N R V P M N S G K I K N R R K A I E 1261 GGATCTGATATGGAAGACTCTGATAGGAAGAAGAATAGCAAGAAAAGCAATTGGA E D S D M E D S D M E D A D T E E K P L A 1321 CATGGTGCTACAGGGCCTCTTAGAACGATGAATCCAACATCTCTGAAGAAAAGCATCGGAA H G A T G P L R T M N P S T S E E T S E 1341 AAAATCCAACTAATGAGGAATAATGAGGAATCCAACATCAACTTCTGAAGAAAACATCGGAA H G A T G P L R T M N P S T S E E T S E 1351 AAAATTCAATCGGAAGAATGAGGAATCTAGCAATCAAAGTCAGTTGATTTATTA K I Q S N E N N E E S S N Q S Q F D L L 1441 TCGTCTTCAACCGGAAGAGGGGGTTAAGGAATTCTGCGAAGGCGGCCTGGGACTTCTTGGT S S S T E E G L R I A L P E P P G L L G 1501 TTCAATATGCAAAATAATGAAGGAGTTGCTCTGCGGGAGTGGGAATGGGACTACTTTTAATCC F N M Q N N E L E S S V S E P S S F N L 1561 TCACTGCCACCCGAAGAGGGCTTACCGGCAATGGGGAATGGCGACTGCTTATCCCCA S S P P T E E E L A A M G I T L S V T P 1621 TCAGTTGAAGAGGAATCGAGCTTGCCCGCCACGGGAAAGGCTTACCGCGAAGGGAT A N P S L E F S S L H L P K E D A P Q S L T 1661 GCTAATCCATCCTGGACATCCCCCCCCACCGGAGAAGAGGTTAGCGCCTAATGGAAT A N P S L E F P S P P T E E L A A M D 1741 ATGAAGCAAAGATATAGCTCCGGACAGGGAGATCCACGAGAGGAGTTGGCGCCAATGGAAT A N P S L E F N P S P T E E L A A M D 1741 ATGAAGCAAAGATATAGCTCCGGACAGTGAGAATCCACCTCAGGCGCAATGGAAT A N P S L E F P S P P T E E L A A M D 1741 ATGAAGCAAAGTATAGCTCCGGACAGTTGAAGGAGATCGAGCTTGCGGCCCAATCGAAT A N P S L E F N P S P T E E L A A M D 1741 ATGAAGCAAAGTATAGCTCCGGCAATCGACCTCATCTAGAGAAA M K Q S I A P T V E G E S S L R P S R E 1861 GAAGAGCTAGCGCCAATGCAATCCAACCATCATCGAGGCTTGCATCCATC	1081	GCTATGCTCGATAGCAGAGCCGAAAAGTAGCAAGGCAAG
1201CAGCAMAATAGAGTACCAATGGAATAGGAAGAGAAAGAAAAGA	1141	GATGAGGTTCCAGCGGTGCTCTTTCACCCCAAACGCAGTATTAAAGAAATATTAAGCGAC D E V P A V L F H P K R S I K E I L S D
1261GGATCTGATATGGAAGACTCTGATATGGAAGATCCTGATACCGAAGAAAAGCCCCTTGG G S D M E D S D M E D A D T E E K P L A1321CATGGTGCTACAGGCCTCTTAGAACGATGAATCCATCAACTTCTGAAGAAAACATCGGAA H G A T G P L R T M N P S T S E E T S E1381ANAATCAATCTAATGAGAATAATGAGGAATCTAGCAATCAAAGTACAAGTATGTTAATTAA	1201	GAGCAAAATAGAGTACCAATGAATAGTAGGTAAGATAAAGAATAGAAGAAAAGCAATTGAA E Q N R V P M N S G K I K N R R K A I E
1321 CATGGTGCTACAGGGCCTCTTAGAACGATGAATCCATCATCATCTTCTGAAGAAACATCGGAA H G A T G P L R T M N P S T S E E T S E 1381 AAAATCGAATCAATCAAGGAATAATGAGGAATCTAGGAATCAAGGTCAGTTTTATTTA	1261	GGATCTGATATGGAAGACTCTGATATGGAAGATGCTGATACCGAAGAAAAGCCGCTTGCG G S D M E D S D M E D A D T E E K P L A
1381 AAAATTCAATCAATGAGAATAATGAGGAATCTAGCAATCAAGTCAGTTTGATTTATTA K I Q S N E N N E E S S N Q S Q F D L L 1441 TCGTCTTCAACCGAAGGGGGTTAAGGATTGCTCTTCCGGGCCCCCTGGACTTCTTGGT S S S T E E G L R I A L P E P P G L L G 1501 TTCAATATGCAAAATAATGAATGGAATCTGGATAGGAACCTAGTCTTTTATTCT F N M Q N N E L E S S V S E P S S F N L 1561 TCATCCCCACCGAAGGAGGAGCTAGCGGCAATGGGGATAACGCTAGTGTTACCCCA S S P P T E E E L A A M G I T L S V T P 1621 TCAGTGGAAGGGATCGGGGCTTGCACTCACCTAAGTGTTACCCCA S V E E E S S L H L P K E D A P Q S L T 1661 GCTAATCCATCGATTGCCACCGCAAGGGGGATAGGGCTTGCGCTCACTCA	1321	CATGGTGCTACAGGGCCTCTTAGAACGATGAATCCATCAACTTCTGAAGAACATCGGAA H G A T G P L R T M N P S T S E E T S E
1441TCGTCTTCAACCGAAGAGGGGTTAAGGATTGCTCTTCCGGAGCCGCCTGGACTTCTTGGT S S S T E E G L R I A L P E P P G L L G1501TTCAATGCCAAATAATAATCAATGGAATCTTCGGATAGGGATAGGACCTAGTCTTTTAATCTC F N M Q N N E L E S S V S E P S S F N L1561TCAATGCCAACCGAAGAGGAGCTAGCGGCAATGGGATAAGGCTAAGTGTTACCCCA S S P P T E E E L A A M G I T L S V T P1621TCAGTTGAAGAGGAATCGAGCTTGCATCTACCTCAAGAGAGAG	1381	AAAATTCAATCTAATGAGAATAATGAGGAATCTAGCAATCAAAGTCAGTTTGATTTATTA KIQSNENNEESSNQSQFDLL
1501 TCCATATATCCAAAATAATCGAATTCGAATCTTCGGTAAGTGAACCTAGTTCTTTTAATCTC F N M Q N N E L E S S V S E P S S F N L 1561 TCATCCCCACCAACCGAACGAGGAGGATCACCGGCAATGGGATAACGCTAAGTGTTACCCCA S S P P T E E E L A A M G I T L S V T P 1621 TCAGTTGAAGAGGAATCGAGCTGCATCTACCTAAAGAAGAGCCCCAGTCGTTAACA S V E E E S S L H L P K E D A P Q S L T 1681 GCTAATCCATCGATCTCCCATCCCCCCCCCCCCCACCGAGAAGAAGTTACCGGCAATGGAT A N P S L E F P S P P T E E E L A A M D 1741 ATGAAGCAAAGTATACCTCCCCACCGAAGAAGAAGTTACCGGCAATGGAAT M K Q S I A P T V E G E S L R P S R E 1801 GATGGCGCTCAGTGGTTAACAGCTAATCCATCTTGGAGTTCCCATCCCACCGAACGAA	1441	TCGTCTTCAACCGAAGAGGGGTTAAGGATTGCTCTTCCGGAGCCCGCCTGGACTTCTTGGT S S S T E E G L R I A L P E P P G L L G
1561 TCATCCCCACCGAACGGAAGAGGAGCTACGGGATAACGCTAAGGCTAACGCAACGCAACGGAAGAGGAGCCCCAGCGAAGGGATACGCCCCAGCCTAACCA S S P P T E E E L A A M G I T L S V T P 1621 TCAGTTGAAGAGGAATCGAGCTTGCATCTACCTAAGAAGATGGACCCCAGTGGTTAACA S V E E E S S L H L P K E D A P Q S L T 1681 GCTAATCCATCATGGAATTCCCATCCCCCCCCACCGAGAGAAGGTTAGCGCCAATGGAT A N P S L E F P S P P T E E E L A A M D 1741 ATGAAGCAAAGTATAGCTCCGACAGTTGAAGGAGAATCGAGCTTGCGTCCATCTAGAGAA M K Q S I A P T V E G E S S L R P S R E 1801 GATGCGCCTCAGTCGTTAACAGCTAAGCAAAGTATGGACTCCCCCCCC	1501	TTCAATATGCAAAATAATGAATTGGAATCTTCGGTAAGTGAACCTAGTTCTTTTAATCTC F N M Q N N E L E S S V S E P S S F N L
1621 TCAGTTGAAGAGGAATCGAGCTTGCATCTACCTAAGAAGATGCACCTCCAGTGGTTAACA S V E E E S S L H L P K E D A P Q S L T 1681 GCTAATCCATCATTGGAATTCCCATCCCCGCCAACCGAAGAAGAGTTAGCGGCAATGGAT A N P S L E F P S P P T E E E L A A M D 1741 ATGAAGCAAAGTATAGCTCCGACGATGAAGAAGAGTTGCGTCCATCTAGAGAA M K Q S I A P T V E G E S S L R P S R E 1801 GATGCGCCTCAGTGGTTAACAGCTAATCCATCATTGGAGTTCCCATCCCCACCGAACCGAA D A P Q S L T A N P S L E F P S P P T E 1861 GAAGAGCTAGCGCAATGGATATGCAACCTAATCACTCGACAGTTGGAGGAGAATCG E E L A A M D M K Q S I A P T V E G E S 1921 AGCTTGCGTCCATCTGGAGAGAGTGCCACTCGGTAACAGCAAAGTATAGCTC F P S P P T E E E L A A M D M K Q S L T A N P S L E 1961 TTCCCATCCCCGCCAACTGAAGGAGAGTGCGCCTCATGGAAGTATGGAAGAAGTATAGCT F P S P P T E E E L A A M D M K Q S I A 2041 CCGACAGTTGAAGGAAATCGAGCTTAGCGCCCCACTCAGAGAAGAAGTATGGCT T A N P S L E F P S P P T E E E L A A M D M K Q S L A 2101 ACAGCTTAACAGCAAAGTATGGAGTCCCCACCCCACGCGAAGGAGTAGGCAAGGTATGGCTAGCAGCAAGGTATGGACTAGCGAGAGAGA	1561	TCATCCCCACCAACCGAAGAGGAGCTAGCGCCAATGGCGATAACGCTAAGTGTTACCCCA S S P P T E E E L A A M G I T L S V T P
1681GCTAATCCATCCATCGCATCCCATCCGCCCACCGAAGAAGAGTAGCGCCAATCGAT A N P S L E F P S P P T E E L A A M D1741ATGAAGCAAAGTATAGCTCCCGACAGTTGAAGGAGAATCGAGCTTGCGCTCCATCTAGAGAA M K Q S I A P T V E G E S S L R P S R E1801GATGCGCCTCAGTCGTAACAGCTAATCCATCATTGGAGTTCCCATCCCCACCCA	1621	TCAGTTGAAGAGGAATCGAGCTTGCATCTAACTAAGAAGATGCACCTCAGTCGTTAACA SVEEESSLHEVPKEDAPOSLT
1741ATGAAGCAAAGTATAGCTCCCACCACCAGTGAAGGACAATCCAGCGTGCGT	1681	GCTAATCCATCGATCGAATTCCCATCCCCCGCCAACCGAAGAAGAGGTTAGCGGCAATGGAT A N P S L E F P S P P T E E E L A A M D
1801GATGCGCTCAGTGTTAACAGCTAATCCATCATTGGAGTTCCCATCCCCACCCA	1741	ATGAAGCAAAGTATAGCTCCGACAGTTGAAGGAGAATCGAGCTTGCGTCCATCTAGAGAA M K O S I A P T V E G E S S L R P S R E
1861GAGAGCTAGCGCATTGGATATGAAGCAAGTATAGCTCCGACAGTTGAAGGGAATCG E1861GAGAGCTAGCGCATTGGATGAGCACAGTAGAGCTCGACCGAC	1801	GATGGGGCTCAGTGGTAACCAGCTAATCCATTGGAGTTCCCATCCCCACCAACCGAA D D O S L T D N P S L E F P S P T F
1921ACCTTCGCTCCATCTAGAGAAGAAGCAGCCTCAGTCGTTAACAGCCAATCCATCATTGGAA S L R P S R E D A P Q S L T A N P S L E1981TTCCCATCCCCGCCAACTGAAGAAGAGTTAGCGGCAATGGATATGAAGCAAAGTATAGCT F P S P P T E E E L A A M D M K Q S I A2041CCGACAGTGAAGCAATCGAGCTTGCGTCCATCTAGAGAAGAAGCGCCCTCAGTCGTTA P T V E G E S S L R P S R E D A P Q S L2101ACAGCTAATCCATCATTGGAGTTCCCATCCCACCCCAACGAAGAAGAGGCGCAATGG T A N P S L E P P S P P T E E E L A A M2161GGTATAAAGCAAAGTATGGCTCTGCAGTGGAGAAGAAGCAGCTGCACCGACCAGGGCAATG T A N P S L E P P S P P T E E E L A A M2161GGTATAAAGCAAAGTATGGCCTTGCAGTGGAGAAGAAGTCGAGCTTGCATCCATC	1861	GAAGAGCTAGCGGCAATGGATATGAAGCAAAGTATAGCTCCGACAGTTGAAGGAGAATCG E E L A A M D M K O S I A P T V E G E S
1981TTCCCATCCCCCCCAACTGAAGAAGATTAGCGCCAATGGATATGAAGCAAAGTATAGCT F P S P P T E E E L A A M D M K Q S I A2041CCGACAGTTGAAGCAAATCGAGCTTGCGTCCATCTAGAGAAGATGCGCCCCAGTCGATT P T V E G E S S L R P S R E D A P Q S L2101ACAGCTAATCCATCGAGTCCCATCCCATCCCACCCAACGAAGAAGAGCTAGCGGCAATG T A N P S L E F P S P P T E E E L A A M2161GGTATAAAGCAAAGTATGGCCTCGCTCCATCTAGAGAAGAACGAGCTGCACCCACC	1 92 1	ACCTTGCGTCCATCTAGAGAAGAAGCAACCCACTCAGTCGTCAACAGCCAATCCATCATTGGAA S L R P S R E D A P O S L T A N P S L E
2041 CCGACAGTGAAGAAGATGGAGATGGAGATGGAGAGAAGATGGGCCTCAGTGGTTA P T V E G E S S L R P S R E D A P Q S L 2101 ACAGCTAATGCATCATTGGAGTTCCCATCCCACCAACGAAGAAGAGGCTAGCGGCAATG T A N P S L E P P S P P T E E E L A A M 2161 GGTATAAAGCAAAGTATGGCTCTGTCAGTGGAGAAGAATCGAGCTTGCATCCATC	1981	TTCCCATCCCCCCCAACTGAAGAAGAGTTAGCGGCAATGGATATGAAGCAAAGTATAGCT F P S P P T E E E L A A M D M K O S T A
2101 ACAGCTAATCCATCGAGGTCCCATCCCATCCCAACGAAGAAGAGCTAGCGGCAATG T A N P S L E F P S P T E E E L A A M 2161 GGTATAAAGCAAAGTATGGCTCTGTCAGTTGGAGAAGAATCGAGCTTGCATCCATC	2041	CCGACAGTIGAAGGAGAATCGAGCTTGCGTCCATCATAGAGAAGATGCGCCTCAGTCGTTA P T V E G E S S L R P S R E D A P O S I
2161 GGTATAAGCAAAGTATGGCTTCTCTCGTGGTGGAGAAGAATCGAGCTTGCATCCATC	2101	ACAGCTAATCCATCATTGGAGTTCCCATCCCCACCAACGAAGAAGAGCTAGCGGCAATG
2221 GAAGATGCCCCTCAGTGGTGAGCGAATCCATCGAGTGCCATCACCACCACCT E D A P Q S L T A N P S L E L P S P T	2161	GTATAAAGCAAAGCATCGCTCTGCTCAGTGGAGAAGAATCGAGCTTGCATCCATC
	2221	GAAGATGCGCTCAGTCGTTAACAGCCAATCCATCGTTGGAGTTGCCATCCCCCCACCAACT E D A P Q S L T A N P S L E L P S P P T

ATTGTTTTCCTAAATTAGCTAATAGGGAGGTTTTAACAAGAGTCAAAAAACTCTAAACAGC

2281	GAAGAAGAGTAGCGGAATGGATATGAAGCAAAGTATAGCTCCGACAGTTGAAGGAGAA $E\ E\ E\ L\ A\ A\ M\ D\ M\ K\ Q\ S\ I\ A\ P\ T\ V\ E\ G\ E$
2341	TCGACCTTCCGTCCATCTAGAGAAGATGTGCCTCAGTCGTTAACAGCCAATCCATCATTG S S L R P S R E D V P Q S L T A N P S L
2401	GAGTTGCCATCCCACCAACTGAAGAAGAGTTAGCGGCAATGGATATGAAGCAAAGTATA E L P S P P T E E E L A A M D M K Q S I
2461	GCTCCGACAGTTGAAGGAGAATCGAGCTTGCGTCCATCTAGAGAAGATGTGCCTCAGTCG A P T V E G E S S L R P S R E D V P Q S
2521	TTAACAGCTAATCCACCATTGGAGTTCCCGTCACCGCCAACTCGAGCAGAATTAGCGGCA L T A N P P L E F P S P P T R A E L A A
2581	ATGGGAATATTTATGTTTAACGACGCCTTATTGACGGGGGACTTAGGAAGTGCCGCGGAAT M G I F M F N D G L L R G D L G S A G N
2641	GCCATAGAGCGACAGAGTTCAAGTTGCCTTGATTGGCCCAGGTGTTCATATTTCTATGGA A I E R Q S S S C L D S P T C S Y F Y G
2701	TTCCATACAGATGATGAGTATAGTGATTCAGAAGACGAGTTAGACAGCCTTTTAAATCCA F H T D D E Y S D S E D E L D S L L N P
2761	aaagtaactgcaattggtgaaactaaaaaacatcaccaacagtatagaaaggtaggattt K V T A I G E T K K T S Q Q Y R K V G F
2821	ATGCCATTTGTCCCTCTTTGTAAAAGCCTTTGCGGGGGGGAAATGGAACTCTTGCAGAG M P F V P L L E K P F A G G N G T L A E
2881	GAGCAGATTATCAAAAATCCACTTGGAAAAACTAAGCAAGAGCTGCAACTCCAAGACAGT E Q I I K N P L G K T K Q E L Q L Q D S
2941	ATAGAGCAGCAATTAACTTTAGCGAAGAAATTACAAGATAACGCTGAAAAAGCTGCTGAA I E Q Q L T L A K K L Q D N A E K A A E
3001	ACAACTGAAGTATCTAAAACAAATGAAACAACTAAAACAAATGAAGTAATTAAAACAAAT T T E V S K T N E T T K T N E V I K T N
3061	GAAACTATTAAAACAGCCATACTCCCCAAAAACCGAACCGGCCTGAAGGTCTATCGCTACTA E T I K T A I L P K T E P A E G L S L L
3121	ACGAATAGTACTCAAACAGTAGGAAGTACGTACGAATAAACTTCATAGT T N S T Q T V G S T L K V S R N K L H S
3181	$\begin{array}{c} ccccaaaaactactggtactttaaaatcagaaaattcttcttcttaataccagctggaatg \\ P \ Q \ K \ T \ T \ G \ T \ L \ K \ S \ E \ N \ S \ S \ L \ I \ P \ A \ G \ M \end{array}$
3241	CCGATTATTCCATTGGAAAGTTTGGAGAAAAGGAAGGAACAGCCGAAAACAAATCTAGCT P I I P L E S L E K R K E Q P K T N L A
3301	GAAGGTACGCTCAAAAATAATAATTAGTAGAAAAGAGTGAAGGTAAAAATACGCCTTCT E G T L K N N K L V E K S E G K N T P S
3361	AGAAGCGGATCGGAAAAATTAGTTGCTAAAAGTGCTGAGCGCGAAAAAACAAATCAAGAA R S G S E K L V A K S A E R E K T N Q E
3421	GCCGGAAACAATACGGTCTTAATGTATGCTTTAGTAGCGATTGGGATTATTTCATTAGCT A G N N T V L M Y A L V A I G I I S L A
3481	GTAATTATTAAGATTATCCGTACAAGAAAAAGTGATTAAAATTTTGAATCGCTTCAGGAA V I I K I I R T R K S D ***
3541	ATAAATGAGAGGAAGAATAATGCTATGAGAATCAACAAAATATCGCTTGGTATATATGTG M R I N K I S L G I Y V
3601	TTTATCTTTGCTTTGACTGCATCTCTGATGAAAGTAAATGCCTGCTCTGATGATCATCTG F I F A L T A S L M K V N A C S D D H L
3661	ATGCAGCCTCAAACACCTAATATAGAACATAAATTACCTCATAAGCTTAGCTGGTCAGCA M Q P Q T P N I E H K L P H K L S W S A
3721	GAACGACCCTTAGACGAAAAGGCTAATACGCATTTATGGTTATTTAAACAAGCAAG
3781	ATATTGGCCAAAGAAAACAATGGCGCGTATAAGGAATTGCTTGAGATGTTAAACAAATAT ILAKENNGAYKELLEMLNKY
3841	TATAAAGAAGTTGCTCAAGGTATATTTGATGCTGACCATAAAAATCCCTATTACGATTGT Y K E V A Q G I F D A D H K N P Y Y D C
3901	AGTACGTTTGTATCGCATTTTTATAACCCAACTAAAGATAATACTTATTTACCAGGTTTT S T F V S H F Y N P T K D N T Y L P G F
3961	AAAAATGCGAAACAGACCTGCGGCAAATATTTTAAACAAGCTTTGCAAGATTATAAAGAG K N A K Q T C G K Y F K Q A L Q D Y K E
4021	GATAAGCTCAATACAGCTTTCTACAAATTAGGCTTGTCCATTACTTAC
4081	SphI AGCCAACCAAT <u>GCATGC</u> S Q P M H

1

61

121

181

ActA

lactA

(Listeria ivanovii)



FIG. 4. Amino acid sequences of IactA of L. ivanovii and ActA of L. monocytogenes. The proline-rich repeat regions are boxed, and the most conserved sequences are highlighted. Numbering starts at the NH₂ terminus of the mature ActA protein and at the putative NH₂ terminus of the mature IactA protein. Hydrophobicity plots are shown under each protein sequence.

protein are hydrophobic, suggesting that this protein has a N-terminal signal sequence and could be targeted to the cell membrane and that the C-terminal hydrophobic region of the protein could act as a membrane anchor. This latter hypothesis is corroborated by the fact that the C-terminal hydrophobic region is followed by positively charged residues which could act as a stop transfer signal. Thus the mature protein is probably 1,044 amino acids long and targeted to the bacterial surface.

The sequence can be artificially divided into three parts. The first (positions 1 to 390) contains a very charged region extending from positions 89 to 219 with two highly charged clusters (positions 89 to 116 and positions 153 to 219). The second and central part (positions 391 to 735) is made of seven and a half highly conserved tandem 47-amino-acid-long proline-rich repeats. The third and C-terminal part extends from residue 736 to the C-terminal hydrophobic sequence. Three short peptides are present twice in the protein sequence, KVEEG at positions 29 to 33 and 183 to 187, SDMED at positions 294 to 298 and 299 to 303, and EV(S/I)KTNET(T/I)KT at positions 875 to 885 and 887 to 897. The amino acid composition of the protein shows no exceptional bias except a relatively high content in cysteine residues. These six cysteines are scattered throughout the sequence. The first two are in the signal peptide, and the second is in a region which presents some homology with lipid attachment sites of lipoproteins. IactA presents several putative phosphorylation sites: two cyclic AMP-dependent phosphorylation sites (at position 149 and 801) and many putative phosphorylation sites for casein kinase II and protein kinase C. Finally, the proline-rich region of IactA displays eight times the consensus sequence X-Ser/Thr-Pro-X for the proline-directed protein kinase (58). The calculated pI of the mature protein is 4.48.

The most closely related protein to IactA is the ActA protein of L. monocytogenes, which is only 610 amino acids long (15, 36, 56) (Fig. 4). ActA and IactA share 34% sequence identity and 52% sequence similarity in an overall alignment. The ActA protein can also be divided in three parts, all of which are shorter than their L. ivanovii counterparts. The central region of ActA contains only four tandem proline-rich repeats which are shorter and less conserved than those of IactA. As shown in Fig. 4, the first part of the proline rich repeats is highly homologous to the N-terminal part of the IactA repeats with a consensus sequence E/D-L/F-S/P-S/P-P-T-E/D-E/D-E-L/F. In contrast to IactA, the mature ActA does not contain any cysteinyl residues. The ActA sequence also contains putative casein kinase II phosphorylation sites, but the proline-rich repeats do not display the consensus sequence of prolinedirected protein kinase (see Discussion).

The protein encoded by *iactA* migrates aberrantly on SDS gels. Preliminary immunofluorescence experiments had indicated that affinity-purified anti-ActA antibodies raised against a peptide of the repeat region of ActA of L. monocytogenes (37) could reproducibly label intracellular L. ivanovii. The la-



FIG. 5. Analysis of iactA genes and iactA gene products. (A) Western blot analysis of bacterial surface proteins using affinity-purified anti-ActA repeat region antibodies (see Materials and Methods). On the same gel, various quantities of total protein extracts were loaded: their relative ratios were 1 for the L. ivanovii strain extracts (BUG497, BUG496, BUG598, and BUG1188), 1/500 for L. monocytogenes LO28, and 15 for LO28DactA and LO28DactA complemented with a plasmid carrying the cloned iactA gene from L. ivanovii BUG497. The membrane was cut in different slices which were incubated in the same antibody preparation and revealed simultaneously in the chemiluminescence mixture but exposed at various times (30 s for the four L. ivanovii extracts, 2 min for LO28, and 2 h and 45 min for the L. monocytogenes DactA strains). The two major IactA-specific bands detected after longer exposure in the complemented strain are indicated by arrowheads. Molecular size markers are indicated in kilodaltons on the left. (B) PCR analysis of the DNA region encoding the repeat region of IactA in different L. ivanovii strains. Oligonucleotides derived from the iactA gene sequence of BUG497 and located on both ends of the DNA region encoding the repeat region of IactA were used in a PCR amplification experiment using chromosomal DNA from four L. ivanovii strains or no DNA in the control. PCR amplification products were analysed by electrophoresis on a 1% agarose gel and ethidium bromide staining. Molecular size markers (lane MW) are indicated in kilobases.

beling on *L. ivanovii* displayed a polarized distribution similar to the distribution of ActA (35, 37) on the surface of *L. monocytogenes* inside infected cells (data not shown).

We used the same antibodies in Western blot analysis to detect IactA in bacterial surface protein extracts. Although the calculated molecular size of IactA is 115 kDa, we reproducibly detected multiple bands, the two predominant of which migrated as approximately 190- and 250-kDa proteins (Fig. 5A). These two bands correspond to the bona fide *iactA* gene product since the same bands were also detected, albeit at a very low level, in an *L. monocytogenes* strain carrying a deletion of *actA* and complemented with the *iactA* gene (see also below and Fig. 5A). This weak signal is probably due to both a low expression of IactA in vitro and the fact that the affinity-purified antibodies raised against a peptide of ActA poorly recognize IactA, in agreement with low sequence similarity in this region of the protein.

The reason for the appearance of two or more bands is unclear. We investigated whether *L. ivanovii* carries a single copy of the *iactA* gene by Southern hybridization under lowstringency conditions of hybridization by using the *SphI* fragment, carrying *iactA* as a probe, on *SphI*-digested *L. ivanovii* chromosomal DNA. Detection of a single band suggested that there is only one copy of *iactA* on the *L. ivanovii* chromosome, although the presence of a second copy of *iactA* present on a *SphI* fragment of the same size cannot be definitively ruled out (data not shown).

Evidence for size variation in various *L. ivanovii* strains. Since variations in the size of ActA have been reported in *L*. *monocytogenes* (46), we investigated whether size variations occur in IactA. We thus performed Western blot (immunoblot) analysis on three other *L. ivanovii* strains including the type strain and a subspecies of *L. ivanovii*, *L. ivanovii* subsp. *londoniensis* (5). This analysis revealed that like the first *L. ivanovii* strain analyzed, the three other strains expressed two or more high-molecular-weight protein bands which reacted with the anti-ActA anti-repeat region antibodies. However, in the different species, these bands differed in size (Fig. 5A).

Since the sequence of *iactA* had revealed a region containing tandem repeats present at both the nucleotide level and the protein level, we examined whether the DNA region containing the repeats varied in length by amplifying this region by using two oligonucleotides derived from the sequence of iactA and present on both sides of the corresponding DNA region. In all four cases, one fragment was amplified (Fig. 5B). These fragments differed in size, the larger ones corresponding to the two strains BUG496 (type strain) and BUG598, which also express the largest detectable protein bands (Fig. 5A). The smallest DNA fragments correspond to the two strains in which the highest detectable bands were smaller (Fig. 5A). From the size of the amplified DNA fragments, we could calculate that compared to BUG497, the strain from which we determined the sequence of *iactA*, the two strains BUG496 and BUG598 probably encode a protein with an additional 47amino-acid repeat. Strain BUG1188 could encode a protein lacking about 47 amino acids, the length of one repeat. Taken together, these data suggest that iactA varies among L. ivanovii strains and that the variation could be due to a variation in the length of the repeat region. However, actin polymerization does not seem to depend on the number of repeats since all three strains were able to induce actin assembly and tail formation (Fig. 1).

Transformation of an L. monocytogenes strain carrying an actA deletion, with *iactA* restores actin polymerization and movement. To establish that *iactA* is playing the same role as actA, we introduced it into an L. monocytogenes strain carrying a large internal deletion of the actA gene (see Materials and Methods). This strain is unable to polymerize actin and is nonmotile in the cytosol of infected mammalian cells. After intracellular multiplication, it gives rise to characteristic microcolonies near the nucleus (Fig. 6B). When this strain was transformed with pIV11, a plasmid carrying iactA under the control of a strong and constitutive promoter, it recovered the capacity to assemble actin and move in the cytosol with the formation of actin tails ressembling those generated by L. monocytogenes and L. ivanovii (Fig. 6C and D). As mentioned above, we detected, by Western blot analysis, two bands in the protein extracts from the complemented strain which comigrated with the protein bands revealed with the anti-ActA antibodies in the L. ivanovii extracts (Fig. 5A). Taken together, these results definitively establish that *iactA* is the L. *ivanovii* functional homolog of actA.

DISCUSSION

L. monocytogenes and L. ivanovii have similar behaviors in infected cells (34). Both species are indeed able to assemble actin and use this mechanism to move intracellularly and spread from cell to cell. However, early studies (14, 24, 34, 56) with the whole *actA* gene of L. monocytogenes as a probe in Southern hybridization studies had suggested that L. ivanovii did not contain any sequence homologous to *actA*, the gene required for actin assembly in L. monocytogenes (15, 36, 56). Using the region encoding the proline-rich repeats of *actA* as a probe, we recently detected in L. ivanovii sequences homol-



FIG. 6. Complementation of the $\Delta actA \ L.$ monocytogenes mutant strain with *iactA*. Vero cells infected with *L. ivanovii* CLIP257 (A), LO28 $\Delta actA$ (B), and the transformed strain LO28 $\Delta actA$ (pIV11) (C and D) were stained with fluorescein isothiocyanate-phalloidin, and an antiserum raised against heat-killed *L. monocytogenes* was revealed with rhodamine-labeled anti-rabbit antibodies. Panel A was photographed only for the actin staining (green). Panels B, C, and D were successively photographed for the actin staining (green) and the bacterial staining, which appears in orange due to the very strong actin staining of the whole cell.

ogous to *actA* (25). Following up on this observation, we report here a full characterization of *iactA*, the *L. ivanovii actA* homolog. The most interesting result of this study is the demonstration that the protein encoded by *iactA*, although not closely related to ActA, can restore actin assembly in an *L. monocy-togenes* mutant strain carrying a total deletion of the *actA* gene. Thus, it provides a new tool to investigate the actin-based motility of *Listeria* spp.

The sequence of *iactA* reveals that the proteins encoded by actA (15, 36, 56) and iactA have a similar overall structure with a N-terminal signal sequence and a C-terminal membrane anchor. These latter features predicted that, similar to ActA, IactA would be a surface protein. This property was demonstrated by immunofluorescence labeling of IactA on the surface of both L. ivanovii and the L. monocytogenes-complemented strain and by the fact that IactA could be extracted by mild SDS treatment. IactA is much larger than ActA (1,044 amino acids for the mature IactA compared to 610 for the mature ActA) but similar to ActA it migrates aberrantly on gels as shown by Western blots with an anti-ActA-specific antibody (37), and in addition it migrates as multiple bands. This last feature may reflect a proteolytic cleavage occurring during the extraction step or a partial posttraductional modification.

The second most striking common feature of both ActA and IactA is the presence of a central region made of proline-rich repeats. However, as shown in Fig. 4, the repeats of IactA are much more conserved and longer than those of ActA. There is evidence that this region in ActA plays a functional and/or structural role (48). It has been suggested that this region in ActA could be a binding site for profilin, a protein involved in the process of actin polymerization, or could be an SH3 (Src homology domain 3) binding site and interfere with transduction pathways leading to cytoskeleton rearrangements (for reviews, see references 11 and 12). Such an interaction remains to be demonstrated. Note that IactA contains in this region eight phosphorylation sites for proline-directed kinase (58), which is not the case for ActA. In the case of ActA, it was reported that phosphorylation of the protein occurs in vivo (8), but the kinase has not yet been identified.

The overall comparison of the two proteins indicates 34% identity and 52% similarity. It is interesting that alignments between ActA and IactA made by using various programs always started at position 161 of IactA. This led us to align the three regions of the two proteins separately. The region of IactA which is the less related to ActA is the N-terminal part (24% identity and 44% similarity when comparing IactA from positions 1 to 390 and ActA from positions 1 to 234). In this case, the sequences aligned by the program Bestfit (13) were regions 161 to 383 of IactA and regions 1 to 234 of ActA. In the same type of analysis, comparison of the central region (region 391 to 736 of IactA with region 235 to 393 of ActA) gave an identity score of 35% and a similarity score of 51%. For the C-terminal region, we obtained an identity score of 32% and a similarity score of 51%. In summary, there seems to be in the N-terminal part of IactA a region which is unrelated to ActA. The rest of the protein is similar to ActA. This lowest degree of similarity in the N-terminal part of the protein is particularly intriguing since we recently showed that the N-terminal part of ActA plays a critical role in the process of actin assembly (17). Also intriguing is the fact that ActA and IactA are strikingly less related to each other than any other proteins present in both L. monocytogenes and L. ivanovii. For example, LLO and ILO share 80% identity (27), the PI-PLC of L. monocytogenes and L. ivanovii share 68% identity, and the two PrfAs share 77% identity (40). Nevertheless, several observations indicate that actA and iactA may be under similar regulatory control mechanisms. We indeed noticed in immunofluorescence studies that the level of expression of IactA was lower after growth in vitro than in vivo, as revealed by the intensity of fluorescence

on bacteria grown in broth medium compared to that of intracellular bacteria in infected cells. A higher expression of ActA inside cells had already been noticed in the case of *L. monocytogenes* and demonstrated to be partly due to the long halflife of the *actA*-specific transcripts and probably also to the stability of the protein as well as to an up-regulation inside cells (6, 8).

The sequence of another *L. ivanovii iactA* gene has been recently determined (37a). This gene encodes a mature protein of 992 amino acids. Compared to the sequence determined here, it lacks 5 amino acids between positions 185 and 189, 47 amino acids between positions 595 and 541, and 1 amino acid at position 524. There is an additional amino acid between positions 263 and 264. This sequence was obtained from the type strain which in our nomenclature is BUG496. This strain produces on Western blot analysis a protein which is larger than the protein encoded by BUG497, the strain that we used to clone *iactA*. We would therefore have expected a larger gene. In addition, we found that the type strain resulted in the largest PCR DNA fragment when we amplified the region encoding the proline-rich repeat region of IactA. The reasons for these discrepancies are unclear.

Searches of the data banks have shown that in contrast to ActA, which is not strongly similar to any protein of the data banks, IactA is significantly similar to MAP1B (47), a 2,464amino-acid mouse microtubule-associated protein (22% identity and 45% similarity when mature IactA is aligned with residues 596 to 1824 of MAP1B) and with the 1,466-aminoacid SPA2 protein of Saccharomyces cerevisiae (22% identity and 45% similarity when mature IactA is aligned with residues 36 to 1453 of SPA2) (20). SPA2 is a protein which colocalizes with the shmoo, the site of polarized cell growth during pheromone-induced morphogenesis, where active actin polymerization takes place. Concerning MAP1B, this protein is associated with microtubules and promotes tubulin polymerization. It is particularly abundant in neurons. The level of MAP1B is highest during the first days of brain development when most neurons are differentiating and sending out processes. There have been some reports in the literature that microtubule-associated proteins interact with F-actin (2, 3, 18, 20, 26, 49), but these ideas are still controversial. In MAP1B, the MAP1B microtubule-binding domain is a basic region containing many repeats of the form KKEE or KKE reminiscent of the charged clusters detected in the first part of IactA (see Results). Whether IactA indeed shares functional homology with MAP1B and whether microtubules play a role in actin-based motility are unknown. It should be noted that phosphorylation seems to tightly regulate the activity of MAP1B. Interestingly, IactA also has many potential phosphorylation sites not only for the proline-directed kinase but also for the cyclic AMP-dependent kinase, casein kinase II, and protein kinase C. Using the sequence of one of the 47-amino-acid repeats of IactA to search data banks, we obtained a high score with the 65-amino-acid F8 surface protein of vaccinia virus (38% identity and 53% similarity) (21). This finding may be significant since it has been clearly demonstrated that vaccinia virus uses the cytoskeleton apparatus to mature virus particles (30, 31, 54, and our unpublished results).

Finally, as for ActA, we did not detect any overall or local sequence similarity between IactA, and IcsA, the protein of the gram-negative bacterium *Shigella flexneri* which plays a similar role in the process of actin assembly (4, 22, 41, 42). Note that these results are not in agreement with the analysis performed by Kreft et al. (37a). The availability of three proteins with apparently the same function will help to understand how a

bacterium can use cellular compounds to move intracellularly and spread from cell to cell.

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