The Major Iron-Containing Protein of *Legionella pneumophila* Is an Aconitase Homologous with the Human Iron-Responsive Element-Binding Protein

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Legionella pneumophila has high iron requirements, and its intracellular growth in human monocytes is dependent on the availability of intracellular iron. To learn more about iron metabolism in *L. pneumophila*, we have undertaken an analysis of the iron proteins of the bacterium. We first developed an assay to identify proteins by ^{59}Fe labelling and nondenaturing polyacrylamide gel electrophoresis. The assay revealed seven iron proteins (IPs) with apparent molecular weights of 500, 450, 250, 210, 150, 130, and 85. IP150 comigrates with superoxide dismutase activity and is probably the Fe-superoxide dismutase of *L. pneumophila*. IP210 is the major iron-containing protein (MICP). To identify and characterize MICP, we purified the protein and cloned and sequenced its gene. MICP is a monomeric protein containing 891 amino acids, and it has a calculated molecular mass of 98,147 Da. Analysis of the sequence revealed that MICP has two interesting homologies. First, MICP is highly homologous with the human iron-responsive element-binding protein, consistent with the hypothesis that this critical iron-regulatory molecule of humans has a prokaryotic ancestor. Second, MICP is highly homologous with the *Escherichia coli* aconitase and to a lesser extent with porcine heart mitochondrial aconitase. Consistent with this, we found that MICP exhibits aconitase activity. In contrast to other aconitases, MICP has a single amino acid change of a potentially deleterious type at a site thought to be critical for substrate binding and enzymatic activity. However, the specific activity of MICP is roughly comparable to that of other aconitases, suggesting that the mutation has at most a mild effect on the aconitase activity of MICP. The abundance of MICP in *L. pneumophila* suggests either that *L. pneumophila* requires high aconitase and perhaps tricarboxylic acid cycle activity or that the bacterium requires large amounts of this protein to serve an additional role in bacterial physiology. A need for large amounts of MICP, which contains four Fe atoms per molecule when fully loaded, could at least partly explain *L. pneumophila*'s high metabolic requirement for iron.

Iron plays a critical role in the immunobiology of the facultative intracellular parasite *Legionella pneumophila*, the agent of Legionnaires' disease. *L. pneumophila* has a particularly high metabolic requirement for iron. Whereas pathogenic bacteria, including gram-negative and facultative intracellular bacteria, commonly require 0.3 to 1.6 μM iron for optimal growth, *L. pneumophila* requires 3 to 13 μM iron for minimal growth and $>20 \mu\text{M}$ iron for optimal growth (14, 26a, 32, 60). In human monocytes, *L. pneumophila*'s growth is strictly dependent on the availability of iron, which it obtains via host iron transport and storage systems, including the transferrin-transferrin receptor system, the lactoferrin-lactoferrin receptor system, and ferritin (4–7). Consequently, agents which reduce intracellular iron availability inhibit *L. pneumophila* intracellular multiplication. These agents include (i) iron chelators, such as the nonphysiologic iron chelator deferoxamine and the physiologic iron chelator apolactoferrin; (ii) the weak bases chloroquine and ammonium chloride, which raise endocytic and lysosomal pH and consequently inhibit the pH-dependent release of iron from endocytized iron transferrin and the pH-dependent proteolysis of ferritin and iron-lactoferrin and release of iron from these molecules; and (iii) gamma interferon, which coordinately downregulates transferrin receptor expression and

intracellular ferritin concentrations (4–7). The inhibitory effects of all these agents on *L. pneumophila* intracellular multiplication in monocytes can be reversed by supplying the host cells with high concentrations of iron in a form in which they can take it up.

Iron is required for the synthesis and activity of a large variety of bacterial iron enzymes and proteins, including heme proteins, iron-sulfur proteins, and iron-binding proteins. The proteins are involved in such diverse functions as electron transfer, oxygen metabolism, peroxide and superoxide metabolism, regulation of gene expression, iron storage, and oxygen binding (45). While much has been learned about the provision of iron to *L. pneumophila* by host cell iron transport systems, little is known about the role of iron in the physiology of *L. pneumophila* or why this organism appears to have such a high metabolic requirement for iron.

To learn more about the role of iron in the physiology of *L. pneumophila*, we have undertaken a study of the iron proteins (IPs) of this microorganism. As a first step, we have developed a method for detecting the major IPs of *L. pneumophila*, taking advantage of the very high sensitivity of iron detection allowed by the use of the radioisotope ^{59}Fe as a label. In this article, we describe this method and show that it detects seven major IPs of *L. pneumophila*, including one in greatest abundance, the major iron-containing protein (MICP). We also describe the purification and partial characterization of MICP and the cloning and sequencing of its gene. We show that MICP is homologous with *Escherichia*

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coli aconitase and the human iron-responsive-element-binding protein (IRE-BP). We demonstrate that MICP has aconitase activity roughly comparable to that of other aconitases, although its sequence has an amino acid change of a potentially deleterious type at a site thought to be critical for enzymatic activity. Finally, we propose that the need for large amounts of this iron-rich molecule could at least partly explain *L. pneumophila*'s high metabolic requirement for iron.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *L. pneumophila* Philadelphia 1 strain was used in all studies. It was grown in embryonated hen eggs, passed once on charcoal yeast extract (CYE) agar (28) at 37°C and 5% CO₂-95% air, and frozen at -70°C in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS; Difco Laboratories, Detroit, Mich.) until used as the starting inoculum in each experiment. In some experiments, *L. pneumophila* was grown in albumin yeast extract (AYE) broth (29) at 37°C with agitation.

E. coli serotype O9:K29:H⁻ (27) was used in the ⁵⁹Fe labelling experiment. Plasmid pUC18 was used to clone DNA fragments in *E. coli* MC1061 [*hsdR mcrB araD139 Δ(ara leu)7679 ΔlacX74 galU galK rspL thi*] (41). Strain MC1061 harboring plasmid pCal3, pCal5, pCal6, or pCal8 was named CAL5, CAL7, CAL8, and CAL10, respectively. *E. coli* strains were grown at 37°C in Luria broth supplemented, when needed, with ampicillin at a concentration of 25 μg/ml in liquid and 50 μg/ml on agar plates.

Chemicals and enzymes. ⁵⁹Fe was purchased as ferric chloride from DuPont NEN (Boston, Mass.), and nitrilotriacetic acid (NTA) (free acid) was purchased from Sigma, Saint Louis, Mo. Porcine heart mitochondrial aconitase and reagents used to detect aconitase and superoxide dismutase (SOD) activities were purchased from Sigma or Boehringer Mannheim Corp. (Indianapolis, Ind.).

Restriction enzymes were purchased from Amersham Corp. (Arlington Heights, Ill.) and New England Biolabs Inc. (Beverly, Mass.); ligase, T4 polynucleotide kinase, and RNase were from Boehringer; RNase-free DNase was from Pharmacia P-L Biochemicals Inc. (Milwaukee, Wis.); and avian myeloblastosis virus reverse transcriptase was from United States Biochemical Corp. (Cleveland, Ohio). [³²P]dCTP (3,000 Ci/mmol) and [³²P]ATP (4,500 Ci/mmol) were purchased from ICN Biomedicals Inc. (Irvine, Calif.), and [³⁵S]dATP (1,000 Ci/mmol) was purchased from Amersham.

Labelling and detection of IPs. *L. pneumophila* IPs were labelled with ⁵⁹Fe by growing the bacteria in the presence of [⁵⁹Fe]NTA on CYE agar plates prepared without ferric pyrophosphate. [⁵⁹Fe]NTA was prepared by mixing a solution of NTA (4.2 mg/ml, pH 7.4) and ⁵⁹FeCl₃ in a 2:1 molar ratio and filter sterilizing it (0.2-μm-pore-size filter). Large inocula of *L. pneumophila* (approximately 2 × 10⁹ CFU per plate) were seeded on plates containing approximately 5 × 10⁷ cpm of label and 25 ml of CYE agar medium, and the plates were incubated at 37°C in 5% CO₂-95% air for 4 days. The bacteria were harvested, washed twice in PBS, sonicated, and centrifuged at 11,000 × g for 10 min, and the supernatant containing the radiolabelled proteins was collected. In subcellular fractionation experiments, periplasmic and cytoplasmic fractions were obtained by successive hypotonic lysis of the outer and cytoplasmic membranes as described previously (55).

Proteins were analyzed by nondenaturing polyacrylamide gel electrophoresis (ND-PAGE) on 7.5% acrylamide gels prepared without sodium dodecyl sulfate (SDS) and β-mercaptoethanol. Gels containing radiolabelled proteins were stained by a modified Coomassie blue staining procedure, avoiding acidic conditions. Gels were incubated for 1 h in staining solution containing 25% methanol, 5% Formalin, and 0.1% Coomassie blue and destained in 25% methanol and 0.37% Formalin. Gels were then air dried between sheets of cellophane. Autoradiography was performed at -70°C with an intensifying screen.

Purification of MICP. The protocol for purifying MICP was established by using ⁵⁹Fe-labelled bacteria, obtained as described above, so that each step in the purification process could be monitored by counting radioactivity and by autoradiography of ND-PAGE gels. The protocol was then applied to unlabelled bacteria grown under the same conditions, i.e., on solid medium not containing supplemental iron. Bacterial growth was observed under this condition only because the use of a very large inoculum (2 × 10⁹ CFU per 100-mm plate) allowed confluent growth to be reached after only a few bacterial doublings, minimizing iron requirements.

MICP was purified from a crude bacterial lysate prepared as follows. The *L. pneumophila* bacteria grown on 100 CYE plates were harvested, suspended in PBS, and sonicated on ice for 12 min with a 2.5-cm-diameter probe in a pulse mode (0.5-s pulse per s) at 175 W with a W-375 model sonicator (Heat Systems Ultrasonics, Inc., Plainview, N.Y.). The lysate (supernatant) was collected by centrifugation at 5,000 × g for 10 min, and the sonication procedure was repeated on the unlysed bacteria in the pellet. Lysates from the two sonication procedures were combined and centrifuged for 1 h at 272,000 × g to pellet membrane debris.

MICP was purified from the bacterial lysate by a three-step procedure involving ammonium sulfate precipitation, ion-exchange chromatography, and molecular sieve chromatography. The bacterial lysate was saturated with ammonium sulfate to a level of 65% for 1 h at room temperature, and the precipitate formed was pelleted by centrifugation at 23,000 × g for 30 min and discarded. The supernatant was brought to 85% saturation with ammonium sulfate and held overnight at room temperature, and the precipitate, containing MICP, was collected by centrifugation at 7,000 × g for 30 min. The precipitate was resuspended in TA buffer (0.05 M Tris-HCl, 1.5 mM NaN₃ [pH 8.4]), applied to a Sephadex G200 or Sephacryl S400 HR column (48 by 3 cm; Pharmacia), and eluted with TA at a flow rate of 30 ml/h (45 ml/h with the Sephacryl S400 HR column). Fractions containing MICP were pooled and loaded directly onto an ion-exchange Q Sepharose fast-flow chromatography column (5.5 by 3 cm; Pharmacia). MICP was eluted with a linear salt gradient (0 to 0.3 M NaCl) in TA buffer. Approximately 1.2 to 2 mg of MICP was purified from the bacteria harvested from 100 plates.

DNA manipulations. Chromosomal DNA was prepared from *L. pneumophila* grown on CYE plates as described previously (42). Oligonucleotides used as sequencing primers or probes were synthesized by the phosphoramidite method on a model 392 DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.) and 5' end labelled when used as a probe. Recombinant DNA techniques were performed as described previously (41). DNA probes were labelled by the multiprime labelling system (kit RPN.160 1Y; Amersham). Southern blot DNA hybridization and colony hybridization were performed with Hybond-N nylon membranes

FIG. 1. Major IPs of *L. pneumophila*. A crude extract of *L. pneumophila* grown on agar medium containing ^{59}Fe was subjected to ND-PAGE, and the radiolabeled proteins were visualized by autoradiography (right). A crude extract of *E. coli* obtained under the same conditions was included as a control (left).

(Amersham). High-stringency hybridization was performed with a rapid hybridization solution (Amersham) as recommended by the manufacturer. For low-stringency hybridizations with oligonucleotides as probes, membranes were incubated overnight at 40°C with the probe (10^6 cpm/ml) in a solution containing 1 M NaCl, 50 mM Tris-HCl (pH 7.4), 1% SDS, 10% dextran sulfate, and 200 μg of sonicated salmon sperm DNA per ml and then washed twice for 30 min in $6\times$ SSC ($1\times$ SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) containing 0.1% SDS at increasing temperatures (40 to 48°C). Low-stringency hybridizations with DNA fragments as probes were performed as described previously (17). The DNA sequence was determined with the Sequenase kit (United States Biochemical).

RNA manipulations. Total cellular RNA was extracted from *L. pneumophila* grown to stationary phase in AYE broth at 37°C with agitation essentially as described previously (13). Northern (RNA) blotting and promoter mapping by primer extension with reverse transcriptase were performed as described previously (42, 43).

Enzyme assays. The SOD activity of proteins analyzed by ND-PAGE was measured as described before (2).

Aconitase was assayed spectrophotometrically at 240 nm by monitoring the formation of *cis*-aconitate from isocitrate as described by Kennedy et al. (34). An activity stain for aconitase in ND-PAGE was performed by coupling the conversion of *cis*-aconitate to isocitrate to a colorimetric test for isocitrate dehydrogenase as described by Prodromou et al. (49).

Sequence analysis. The data bases of the University of Wisconsin Genetics Computer Group were searched with the Fasta and TFASTA programs. The presence of putative open reading frames with a high probability of being coding sequences was screened for with the program Codon Preference (12).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned accession number L22081.

RESULTS

Identification of the major iron-containing proteins of *L. pneumophila*. We identified the major IPs of *L. pneumophila* by using ^{59}Fe as a marker and ND-PAGE followed by

FIG. 2. Analysis of purified MICP. Purified MICP (5 μg) was subjected to SDS-PAGE. The gel was stained with Coomassie blue. MICP contains a single subunit that has an apparent molecular mass of approximately 98 kDa. DF, dye front.

autoradiography. The use of nondenaturing conditions was essential because the IPs retained their iron label under such conditions. With this method, we detected seven IPs with molecular masses of approximately 500, 450, 250, 210, 150, 130, and 85 kDa (Fig. 1). The major one had an apparent molecular mass under nondenaturing conditions of 210 kDa and was named MICP (for major iron-containing protein). While ND-PAGE provided a convenient and reproducible reference system for the major iron-containing proteins of *L. pneumophila*, it did not provide an accurate determination of molecular mass. Subsequent analysis demonstrated that MICP has a molecular mass of 98 kDa (see sequence analysis below).

The pattern of labelled proteins obtained with *L. pneumophila* differs from that obtained under the same conditions with *E. coli*. Only the major IP of *E. coli*, probably bacterioferritin from its very high molecular mass and intense labelling, comigrates with an IP of *L. pneumophila*, that of 450 kDa (Fig. 1).

A subcellular fractionation study showed that all seven IPs are in the cytoplasmic-periplasmic fraction; none were clearly detected in the membrane fraction (data not shown). Homology data presented elsewhere in this article indicated that MICP is a cytoplasmic protein. This was confirmed by immunogold ultracyromicroscopy of *L. pneumophila* with monospecific polyclonal antibodies raised against purified MICP (8a).

Cell extracts of *L. pneumophila* are known to contain several SOD activities, but the metal cofactor of the various SODs has not been determined (47). Using an assay for detection of SOD activity of proteins in nondenaturing gels (2), we found that the IP with an apparent molecular mass of 150 kDa (IP150) comigrates with the fast-moving SOD of *L. pneumophila* (data not shown). Therefore, IP150 is probably an iron-containing SOD.

Aside from IP150, the identities of the other IPs of *L. pneumophila* were not apparent. To identify them, we first focused on MICP.

Purification of MICP, the major iron-containing protein of *L. pneumophila*. We purified MICP from sonicates of bacteria grown on CYE plates prepared without iron supplement-

tation by a three-step procedure: ammonium sulfate precipitation, ion-exchange chromatography, and molecular sieve chromatography. We used ^{59}Fe -labelled MICP to develop the purification protocol, and subsequently we purified MICP without any radiolabel present. We analyzed purified MICP by PAGE under nondenaturing (data not shown) and denaturing (Fig. 2) conditions. The apparent molecular masses of the purified protein as analyzed by SDS-PAGE (85 kDa) and by gel filtration (90 kDa) were similar, indicating that MICP is a monomeric protein.

Cloning of *acn*, the MICP-encoding gene. As the NH_2 -terminal residue of MICP was not accessible to Edman degradation, we determined the NH_2 -terminal sequence of three internal peptides of the purified protein obtained by cyanogen bromide cleavage. These sequences were named AA1, AA2, and AA3. We synthesized an oligonucleotide (JM01) derived from AA1, introducing inosine residues at nearly every degenerate position (Fig. 3).

Under low-stringency conditions of hybridization, Southern blot analysis of total chromosomal DNA restricted with various enzymes revealed a 6-kb *Pst*I fragment which hybridized with the 5'-end-labelled oligonucleotide JM01. We used this oligonucleotide to screen a bank of chromosomal *Pst*I fragments cloned in pUC18 and selected two positive clones, each harboring a plasmid that contained the 6-kb *Pst*I fragment in addition to other *Pst*I fragments. We subcloned the desired 6-kb *Pst*I fragment in pUC18 and named the resulting plasmid pCal3 (Fig. 4). We also subcloned in pUC18 the 1.5-kb *Hind*III-*Pst*I fragment from pCal3 that hybridized with oligonucleotide JM01 and named the resulting plasmid pCal5 (Fig. 4). We determined the sequence of its insert on both strands of the DNA. The amino acid sequence derived from the nucleotide sequence contained the internal sequence AA1, showing that the insert of pCal5 was internal to the MICP-encoding gene. We obtained the sequence of the 5' part of the gene from plasmid pCal3. It contained the nucleotide sequence encoding AA3.

Since neither pCal3 nor pCal5 contained the 3' part of the gene, we cloned the 3' part of the gene in two plasmids, pCal6 and pCal8. Plasmid pCal6 was obtained by screening a bank of chromosomal *Hind*III fragments cloned into pUC18 with a 0.9-kb *Eco*RI fragment derived from pCal5 as a probe. Plasmid pCal6 was obtained by screening a bank of chromosomal *Pst*I fragments cloned into pUC18 with a 0.4-kb *Pst*I fragment derived from pCal6 as a probe. We then obtained the sequence of the 3' part of the MICP-encoding gene from plasmids pCal6 and pCal8. This part of the gene contained the nucleotide sequence encoding AA2. The complete nucleotide sequence of the MICP-encoding gene, named *acn*, and the deduced amino acid sequence are presented in Fig. 3.

To determine the number of copies of the *acn* gene and whether related genes are present in the chromosome of *L. pneumophila*, we analyzed the genome in Southern blot experiments under low-stringency hybridization conditions. The 0.9-kb *Eco*RI fragment derived from pCal5 and used as an internal probe for *acn* hybridized to only one fragment when chromosomal DNA was restricted with enzymes that do not cut within the probe (*Eco*RI, *Bam*HI, *Pst*I, and *Hind*III). This demonstrated that only one copy of the *acn* gene was present in the chromosome (data not shown).

Sequence analysis. The open reading frame (ORF) encoding MICP consists of 896 codons (positions 829 to 3516, Fig. 3). The first ATG codon (position 844), located six codons downstream from the beginning of the ORF, is the translation initiation codon, as it immediately precedes the nucle-

otide sequence encoding the experimentally determined amino acid sequence AA3 and is preceded five bases upstream by a putative ribosome-binding site (GGAG). MICP thus contains 891 amino acids and has a calculated molecular mass of 98,147 Da.

We screened for the presence of putative ORFs upstream and downstream from *acn* with a high probability of being coding sequences with a program based on codon usage (see Materials and Methods), taking *acn*, *msp* (3), *ompS* (26), *htpA* (25), *htpB* (25, 53), *pplA* (40), and *mip* (13) as the reference genes. We detected two such ORFs, in the same orientation as *acn*. If the ORF located upstream of *acn* is transcribed and if the first ATG codon is the translation initiation codon, it codes for a protein of 208 amino acids presenting no homologies with known proteins. The 3' end of the ORF located downstream of *acn* is not present on the insert of plasmid pCal8 (Fig. 3).

The transcriptional start of the *acn* gene was determined by primer extension analysis with RNA extracted from bacteria in the stationary phase. Transcription of *acn* starts predominantly at an A in position 810. We also detected a weaker signal indicating a minor transcriptional start at an A in position 828 (Fig. 5). We detected no longer transcript which might have started at a putative promoter identified in the sequence and located upstream of the ORF preceding *acn*. The -10 and -35 regions of the *acn* promoters are similar to the *E. coli* consensus sequences (44).

We identified only a few dyad symmetries in the sequence that would allow the formation of stem and loop structures on the mRNA, but these were of relatively low stability ($\Delta G \geq -14.5$ kcal) (Fig. 3). One dyad symmetry located just downstream of the major *acn* promoter could be involved in regulation of *acn* expression or could be a rho-independent transcriptional terminator for the ORF located upstream of *acn*. We did not detect a good candidate for a rho-independent transcriptional terminator of the *acn* gene in the sequence. This is in agreement with preliminary Northern experimental data showing that *acn* would be transcribed with other downstream genes (data not shown).

A search revealed two putative sites for Fur, the ferric uptake regulatory protein, upstream of the *acn* gene. Each site is located on a different strand of the DNA and has six mismatches in comparison with the consensus sequence (GATAATGATAATCATTATC [1]). One site is located immediately downstream of the major *acn* promoter and overlaps the perfect dyad symmetry mentioned previously (Fig. 3).

MICP is homologous with *E. coli* aconitase and the human IRE-BP. Comparison of the MICP amino acid sequence with those of proteins in the GenBank data base revealed striking homologies with two other proteins, the *E. coli* aconitase (48) and the human IRE-BP (24, 52). These proteins were recently shown to be part of a family of related proteins, including porcine heart mitochondrial aconitase, *Saccharomyces cerevisiae* aconitase, and isopropylmalate isomerases from *Phycomyces blakesleeianus*, *S. cerevisiae*, *Mucor circinelloides*, and *Salmonella typhimurium* (22, 48, 52).

Aconitase is an enzyme [citrate (isocitrate) hydrolyase, EC4.2.1.3] containing a single Fe-S cluster and catalyzing the stereospecific dehydration-rehydration of citrate to isocitrate via *cis*-aconitate in the second and third steps of the Krebs cycle. The enzymatically active form contains a [4Fe-4S] cluster. Under oxidative conditions, aconitase may lose one iron atom, and in the [3Fe-4S] state, it lacks enzymatic activity. Reloading the [3Fe-4S] cluster with iron in vitro restores enzymatic activity. This reversible binding

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	52
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2041	TTA	ATT	GAA	GTT	AAA	GAA	AAA	GAG	AAA	GAA	AAA	ACC	TTT	GCT	GTT	AAA	AAC	AAA	GAT		
	leu	ile	gln	val	gln	lys	gln	lys	gln	lys	gln	acc	thr	phe	ala	val	lys	asn	lys	asp	419
2101	TTT	CAA	ATG	AAA	GAT	GGT	GAT	GTA	GTT	AAA	GCG	ATA	ACC	ACC	TGT	ACT	ACC	ACC	TCT		
	phe	gln	met	lys	his	gln	his	val	ile	ala	ile	thr	ser	cys	thr	asn	thr	asn	thr	asp	439
2161	AAT	GCA	AGT	GTA	CTA	GTA	GCA	GCA	GGA	CTG	GTA	GCA	AAA	GCG	ATT	GAG	AAA	GAA	TTA		
	asn	pro	ser	val	leu	met	ala	ala	gln	leu	val	ala	lys	lys	ala	ile	gln	lys	gln	leu	459
2221	CAG	COC	AAA	CCC	TGG	TTG	ATG	TCT	TCA	TTC	GCA	CCG	TCT	AAA	GTA	GTT	ACT	GAC	TAT		
	gln	arg	lys	pro	thr	val	lys	arg	ser	leu	ala	pro	gln	ser	lys	val	val	thr	asn	thr	479
2281	TTA	AGC	CAT	GCC	GCC	CTA	CAA	ACC	TAC	CTG	GAT	GAA	CTG	GGT	TTT	ATA	TTC	GTT	GTC		
	leu	arg	his	ala	gln	gln	thr	ala	gln	asp	gln	ala	lys	thr	phe	asn	leu	val	gln	thr	499
2341	GCG	TGT	ACC	ACT	TGT	ATA	GTT	AAT	TCG	GGT	CCT	TTC	GAT	GAT	ATT	TCC	CAT	TTC	GTT		
	gln	cys	thr	thr	cys	ile	gln	asn	ser	gln	pro	leu	pro	asp	asp	ile	ser	his	cys	val	519
2401	CAG	CAG	CAT	GAT	CTT	GTT	GTT	TCA	TCT	GTA	CTG	TCT	GCT	AAT	AGA	ATA	TTC	GAA	GCG		
	ala	gln	his	ala	gln	val	ala	asn	ser	val	leu	gln	phe	asn	arg	asn	phe	gln	gln	arg	539
2461	GTC	CAG	COC	CAA	GTC	AGA	COC	AAC	TTC	CTG	GCT	TCT	CCT	GAA	ATA	GTA	GTC	GCT	TAT		
	val	his	pro	gln	val	arg	ala	asn	asn	trp	leu	ala	ser	pro	pro	leu	val	ala	tyr	ala	559
2521	CTA	TCC	GCT	ACA	ACT	TGC	AOC	GAT	ATP	ser	asp	arg	arg	ala	gln	gln	asp	lys	gln		
	leu	cys	gln	thr	thr	arg	arg	asn	ser	leu	ser	arg	gln	pro	ile	gln	asn	lys	gln	asp	579
2581	AAC	GAT	GTT	TAT	CTC	GAA	GAT	ATC	TGC	CCA	TCT	AAT	GAA	GAG	ATT	CGC	GAA	GTC	ATC		
	asn	asp	val	tyr	leu	lys	asp	ile	trp	pro	ser	asn	gln	gln	ile	ala	gln	gln	val	ala	599
JM01								GI	AAI	GAA	TAI	CGI	GAA	GTT	TTT	TGG	GGI	GAT	CGI	ACI	
2641	AAA	GTA	AGC	GGT	ACC	ATT	TTC	CGT	AAA	GAG	TAT	GCT	AAA	TTT	AAA	AGA	GAT	GAT	GAT	CAT	
	lys	val	ser	ser	gln	thr	met	arg	lys	gln	lys	thr	ala	gln	val	phe	lys	gln	asp	ala	619
							F	R	K	E	Y	A	E	V	F	W	G	D	A	T	AA1
2701	TGG	CAA	G																		
	trp	gln	ala	GCC	ATT	CAC	ATC	GCA	GGG	CAA	ACT	TAT	GTA	TGG	AAT	ACT	pro	GAT	TCT	ACA	TAC
	W	Q	A	A	I	Q	T		S	G	P	G									639
2761	ATA	GAA	CAC	CCC	CCA	TTT	GTT	AAA	AAT	TTA	AGT	CTG	AAA	CCA	GAA	CCG	CTC	AAA	CCT	ATC	
	ile	gln	his	pro	pro	phe	phe	gln	asn	leu	ser	leu	lys	pro	gln	pro	leu	lys	pro	ile	659
2821	ATG	CAG	GCT	TAT	GTT	TTC	GCA	CTA	TTC	GCC	GAT	TCA									

FIG. 3. Nucleotide sequence and deduced amino acid sequence of the *acn* gene region. Nucleotides and amino acids are numbered at the left and right sides of the sequence, respectively. *Hind*III and *Pst*I restriction sites are underlined and indicated above the nucleotide sequence. Amino acid sequences deduced from the nucleotide sequence of the *acn* gene and the two bordering ORFs are shown in the three-letter code under the nucleotide sequence. The positions and amino acid sequences of AA1, AA2, and AA3, the three internal amino acid sequences determined by Edman degradation, are indicated in the one-letter code beneath the MICP amino acid sequence. The nucleotide sequence of JM01, the degenerate oligonucleotide used to clone *acn*, is shown above the nucleotide sequence at positions 2663 to 2707. Putative ribosome-binding sites are indicated in boldface characters. Several dyad symmetries are indicated by solid arrows beneath the nucleotide sequence, and the two putative Fur binding sites are indicated by dotted lines above the nucleotide sequence. A putative promoter (nucleotide positions 61 to 101) and the promoters of *acn* (nucleotide positions 775 to 810 and 792 to 828) are indicated by dashed arrows beneath the nucleotide sequence (====->).

FIG. 4. Restriction map and structural organization of the *acn* gene region of *L. pneumophila*. Plasmids used to clone and sequence the *acn* gene region of *L. pneumophila* are depicted. The thick line represents the pUC18 vector, and the thin line represents the chromosomal DNA fragment cloned into it. Apart from the *EcoRI* site, marked with a boldface character and used in the preparation of the 0.9-kb *EcoRI* fragment from pCal5 used as a probe in Southern analysis, only restriction sites of the inserts are indicated (P, *PstI*; H, *HindIII*; E, *EcoRI*). The *EcoRI* site marked with an asterisk may not be the only *EcoRI* site present in the insert of plasmid pCal3. The positions of the *acn* gene of *L. pneumophila* and the two ORFs identified upstream and downstream of *acn* are represented with arrows under the restriction map. The genetic organization of the *acn* gene of *E. coli* is also shown (from reference 48).

of iron in the Fe-S cluster has been proposed as a mechanism for the sensing of intracellular levels of iron (34).

IRE-BP is a cytosolic protein that binds to iron-responsive elements (IREs) under low-iron conditions. IREs are RNA stem-loop structures in the 5' untranslated region (UTR) of ferritin mRNA (18), the 3' UTR of transferrin receptor mRNA (8, 37), the 5' UTR of murine and human erythroid δ -aminolevulinic acid synthase mRNA (10, 11), and the 5' UTR of mitochondrial aconitase mRNA (11, 62). In the case of ferritin mRNA and transferrin receptor mRNA, the interaction of IRE-BP with mRNAs corresponds to a translational regulation of gene expression (36, 57). IRE-BP has

FIG. 5. Mapping of *acn* transcripts by primer extension analysis. A 32 P-end-labelled oligonucleotide primer corresponding to positions 953 to 969 in Fig. 3 was used in the primer extension study. The DNA products were separated by electrophoresis on a 6% polyacrylamide gel (lane 1) simultaneously with the dideoxy sequencing products from a DNA template that contained the 5' part of the *acn* gene (lanes G, A, T, and C, corresponding, respectively, to the dideoxynucleotide used to terminate each reaction). Arrowheads indicate the major and minor transcriptional start sites. The arrow on the right indicates the translational start site of the *acn* gene.

aconitase activity (33). The two activities (aconitase and IRE binding) are exclusive and depend on the iron availability in the cell (19, 20). When its Fe-S cluster is fully loaded, IRE-BP exhibits aconitase activity, whereas in the iron-depleted state, it exhibits IRE-binding activity. Thus, IRE-BP has been proposed to be an iron sensor whose RNA-binding activity is modulated by the intracellular availability of iron (9, 19, 20, 52). The mitochondrial aconitase, as opposed to IRE-BP, the cytosolic aconitase (35), does not possess IRE-BP activity (33).

An alignment of the sequence of MICP with those of *E. coli* aconitase, human IRE-BP, and porcine heart mitochondrial aconitase is presented in Fig. 6. This alignment is based on the alignment of *E. coli* aconitase with the other homologous proteins (48) and shows that *L. pneumophila* MICP is most closely related to *E. coli* aconitase (66% identity). MICP and *E. coli* aconitase are both more closely related to human IRE-BP (52% identity) than to mitochondrial aconitase (28 and 29%, respectively).

Crystallographic structure analysis and mutational analysis of the mitochondrial aconitase have led to the identification of 23 residues contributing to the active site (39, 50, 51, 61). Among those 23 residues, which are involved in substrate recognition, Fe-S cluster ligation and interaction, catalysis, or hydrogen bonding that supports the active-site side chains, only two suffer replacement. Residue A74 of porcine mitochondrial aconitase is replaced by phenylalanine in all three of the other sequences. Most interestingly, residue R580 of porcine mitochondrial aconitase is conserved in all sequences except that of MICP, in which it is replaced by a lysine (K696) (Fig. 6). This residue has been shown to be crucial for substrate binding, as its mutagenesis to a lysine in porcine mitochondrial aconitase resulted in a 30-fold increase in the K_m for isocitrate, a nearly complete loss of tight substrate binding, and subsequently a 250- to 30,000-fold decrease in enzymatic activity (61). We confirmed that the K696 substitution (AAA in the DNA sequence) in MICP was not a cloning artifact or a sequencing mistake by directly sequencing a DNA fragment obtained by polymerase chain reaction amplification of chromosomal DNA (data not shown).

MICP exhibits aconitase activity. The high homology of MICP with aconitases suggested that it might have aconitase enzymatic activity. To explore this, we assayed MICP for aconitase activity by two methods, one for MICP in solution (Fig. 7A) and one for MICP in a gel (Fig. 7B). Both assays demonstrated that MICP exhibits aconitase enzymatic activity. Early in the reaction, the assay used to measure aconitase activity of MICP in solution showed an exponential rather than a linear increase in *cis*-aconitate formation over time. The maximal specific activities of MICP in solution at the two lower concentrations studied, 2.4 and 4.8 μ g/ml, were 15 and 16.5 U/mg, respectively (mean, 15.8 U/mg). The specific activities of MICP and porcine mitochondrial aconitase could not be compared in these studies because the commercially obtained porcine aconitase was relatively impure and not prepared in the same way for the enzymatic studies.

The high homology of MICP with IRE-BP suggested that MICP may have RNA-binding activity. However, using a gel retardation assay, we could detect no specific binding to stem-loop structures homologous with eukaryotic IREs (data not shown).

L.pneu MICP 1 M K V G Q D S L S T K S Q L T V D - - - - - G K T Y N Y Y S L K E A E N K - H F K G I N R - - L P
E.coli ACN 1 - M S S T L R E A S D T Q A K - - - - - D H P L A K S - L G D - T - -
 Hum. IRE-BP 1 - - - - - M S N P F A H L A E P L D P V Q P G K K F F N L L E D S R Y G - -
 Porc. mito. 1 - - - - - R A K V A M S H F E P H E I R D L K N I D I V R - R L N R P T

42 Y S L K V L L E N L L R F E D G N T V T T K D I K A I A D W L H N K T S Q H - E I A F R P T R V L M
 40 K W Q S E E H L G K A H A D R - Y A
 37 F I R A A I N C E F L K K Q E N L H N V T Q H - K N I V P K A I L
 38 L E I V Y G - - H L D P A N Q E I E R G - - - T Y R L R - - - - - D A

91 Q D F T G V P A V V D L A A M R T A I V K M G G N A D K I S P L S P V D L V I D H S V M V D K F A S
 89 E V K R L D T A V N T R G D
 86 F D V K L D P E N V C A I Q F N R R
 72 A A Q M N L Q F I S S G L P K A V - - - - - S T I H C L I E A Q L - -
 ▲ ○

141 A D A L E V N T K I E I E R N K E R Y E F L R W G Q K A F S N F Q V V P P G T G I C H Q V N L E Y L
 139 D E F E V R L M H V K K Q R S
 136 S Q K Q D L F R F K S Q H M R I I S I I I N -
 108 - - G G K D L R R A K D I Q V N A T A G A K Y G - V G F W R S I I I N -
 ▲

191 G K T V W N S E N D G O L Y A Y P D - T L V G T D S H T T M I N G L G V L G W G V G G I E A E A A M
 189 A S E L Q E W I -
 186 A R V F D - - Q - - Y - S D I V
 154 - - - - - G V L I P N G G G I C I A D V D V
 ▲▲▲

240 L G Q P V S M L I P E V I G F K L S G K L K E G I T A T D L V L T V T O M L R K K G V V G K F V E F
 238 D V T R H
 231 I V L Q Y R M P H P L V S I I K H Q V
 191 A I W E L K C K V T S S G W T S P K V I K A G I T V G T A I Y

290 Y G P G L N D L P L A D R A T I S N M A P E Y G A T C G F F P V D K E T I K Y L E L T G R D K H T I
 288 D D S A S I A V L D M R S S E D Q V
 281 F V A Q S I A C A A E V S T V Q E E K L
 241 H V D S I S C T G M C G A I T S V Y N H R M K S K A D I A -
 ◆ ▲

340 A L V E A Y A K A Q G M W Y D K - - - - - D N E E P V F T D S L H L D L G S V E P S L A G P K R P Q D K
 338 E K - - R N - - - P G D I S T E M N D A R
 331 K Y I K K L Q V F - R D F N D P S Q D Q V V E K T V C C S
 290 N A D E F - - - - - K D H L V P D P G C H Y D Q V I E I N S E L K H I N F T D L A

387 V N L S S L P V E F N N F L I E - - - - - V - G K E - K E K E K T F A V K N K D F O M K H G H
 384 A P D V K A A A S - - - - - N E L E N A T H K D R Q P V D Y V M N G H Q Y L P D A
 380 A V D M K K D E S C G A K Q G F K G F Q - A P H H N D H I Y D T E T L A S
 332 H P V - - - - - A V G S V A E K E G W P L D - - -

427 V V I A A I T S C T N T S N P S V L M A A G L V A K K A I E K G L Q R K P W V K S S L A P G S K V V
 427 L V T L K Q A
 429 M L G L V D A N V M Y I T S G
 350 I R V G L G S Y E D M G R S A V - Q L A H K C - - S Q F T I T E Q I

477 T D Y L R H A G L Q T Y L D Q L G F N L V G Y G C T T C I G N S G P L P D D I S H C V A E H D L V V
 477 S A K K T P E P E T A I K K G T
 479 Y Q E S V M P S D V M Q W D R - - - - - K D I K K G E - K N
 397 R A T I E R D Y A Q V R D V G I V L A N A G P

527 S S V L S G N R N F E G R V H P Q V R A N W L - A S P P L V V A Y A L C G T T C S D L S R E P I G Q
 527 G A I L K T - A N M N I N A S H
 529 V G N T Y - I I A I R I F E K L V
 440 T I T Y T N D A N P E T H A F V T E I T L I A L K F N P E T D F L - T
 ◆▲

576 D K E G N D V Y L K D I W P S N E E I A A E V A - - K V S G T M F R K E Y A E V F K G D A H W Q A I
 576 R K D P A Q R A E - - Q T E T E K G
 578 N A K Q Q F T R D - Q A E R Q Y I P G K E V Q K I E T V N E S N L
 489 G D K K F K E A - - D A D L P R A - - - - - E F D P G Q D T Q H P P D S S G Q - R V

624 Q T S S G Q T Y E W N P D S T Y I Q H P P F F E N L S L K P E P L K P I K Q A Y V L A L F G D S I T
 624 N V T R S D G Q E R L S D E M Q A T A V E D H G R I M L V
 627 A P D K L F F S K K S T D L Q P S V D L N L V
 530 D V P T S - - - - - Q R L Q L L E - - - - - D K W D G K D L E D L Q I I K V K G K C

674 T D H I S P A G S I K A S S P A G L Y L K S K G V D E K D F N S Y G S R R G N H E V M M R G T F A N
 674 P D R Q G R E R
 677 N A R N A R T N R L T P R E D A A
 567 A - - - - - P W F R H L D N I S N L - - - - - L I G A
 ○○ ○

724 I R I R N E M T P G Q E G G V T R Y V P T G E T M S I Y D A A M R Y Q E N Q Q D L V I I A G K E Y G
 724 V V M H L D S D V V K Q E T P A V
 727 L L R F L - N K Q A P Q I H L S I L D V F E Q A G L P I V L
 595 N E R - - - - - K A N S V N A V Q F G P V P T R Y K Q H G I R W V G D E N

774 T G S S R D W A A K G T N L L G V K A V I T E S F E R I H R S N L I G M G I L P L Q F N E G T T R K
 774 S P R I R V A Y V V I E P Q V
 776 A P F I L A Y V V I E Y L P E N A D
 640 E E H R L E P R H G R I K A E T K K Q L T A D P A D Y N
 ▲▲▲

824 T L K L D G S E R I S I E I S D K L T P G A M V P V T I E R Q D G D I E K I E T L C R I D T A D E L
 824 G T E K D G D L Q N Q T L T A S Q V V P C R T
 826 A G T Q Y T I P E N K Q M K Q K L D T G K - - - T F Q A V M F D V
 690 K I - - H P V D K L T Q G L K D F A K P L K C I K H P N T Q T L L N H T F N - E T Q I

874 E Y Y K N G G I L Q Y V L R K I S S
 874 T Q D H I N M L K
 872 T F L N M I M A K
 737 W F R A S A N R M K E L Q Q K

DISCUSSION

In this article, we report the detection of seven IPs of *L. pneumophila*. Our assay for detecting IPs used ^{59}Fe labelling and autoradiography of proteins subjected to electrophoresis under nondenaturing conditions. The use of ^{59}Fe gave high sensitivity to the detection assay. By comparison, using a previously described protocol for the specific and nonradioactive staining of IPs in nondenaturing gels (data not shown) (38), we were able to detect only three to four of the seven major IPs detected with our assay. The use of nondenaturing conditions for gel electrophoresis was also a key factor in the detection of the IPs, as even mild denaturing conditions (37°C instead of 100°C) in SDS-PAGE resulted in a loss of or great decrease in the intensity of the autoradiographic signals.

A large number of IPs in bacteria have been identified (45), but our assay, while highly sensitive, still detects only seven IPs in *L. pneumophila*. Presumably, our assay detects those proteins with large amounts of iron per molecule or those in greatest abundance. MICP fulfills both conditions. On the basis of its homologies with other aconitases, MICP has a relatively high iron content, as it contains 4 atoms of iron per molecule in its Fe-S cluster when fully saturated. On the basis of Western immunoblot analysis with purified MICP as a standard, we estimate that *L. pneumophila* has approximately 10^5 MICP molecules per bacterial cell (data not shown), a very high concentration, comparable to that of the major outer membrane proteins of gram-negative organisms (21). Consistent with this, we readily isolated milligram quantities of MICP from relatively small quantities of bacteria.

We show in this study that one of the seven major IPs of *L. pneumophila* is probably an iron-containing SOD. The iron SOD gene of *L. pneumophila* has recently been cloned (54). Aside from this protein and MICP, the identities of the other *L. pneumophila* IPs are not known.

Our study demonstrates that *L. pneumophila* MICP is highly homologous with and therefore closely related to *E. coli* aconitase. The two IPs have the same size, possess the same enzymatic (aconitase) activity, and exhibit 66% amino acid sequence identity. Nevertheless, the proteins of the two species and the *acn* gene regions encoding them exhibit noteworthy differences. First, the formyl methionine of MICP, in contrast to *E. coli* aconitase, is not removed posttranslationally, as determination of the NH_2 -terminal sequence of MICP required cyanogen bromide cleavage of the protein. Second, at the genetic level, the structural organization of the *acn* gene regions of the two proteins differs. The intergenic region located upstream of *acn* is much smaller in *L. pneumophila* than in *E. coli* (84 bp versus 1 kb), and the two upstream genes are not homologous to each other. The intergenic region located downstream of *acn* is much smaller in *E. coli* than in *L. pneumophila*, and the downstream genes are in opposite orientation in the two bacterial species (Fig. 4). Finally, at the transcriptional level,

the *L. pneumophila acn* is probably the first gene of an operon, whereas the *E. coli acn* probably forms a monocistronic transcriptional unit (48). In both cases, Fur boxes are present upstream of *acn*, indicating possible regulation of *acn* expression by the Fur protein (or its homolog in *L. pneumophila*). The significance of these Fur boxes in *L. pneumophila* is questionable, as one was found immediately downstream of the *acn* promoter and one was found divergently oriented in the middle of the upstream ORF. Furthermore, in preliminary studies, we found no evidence that *acn* expression is iron regulated (data not shown). Nevertheless, *acn* is likely to be subjected to the same kind of regulation as the genes encoding the other enzymes of the Krebs cycle or the *E. coli sodA* gene, encoding the MnSOD, which prevents inactivation of aconitase under oxidative attack (15). These genes are controlled by the Arc (aerobic respiration control) regulation system (30, 31, 56). Interestingly, the *L. pneumophila acn* gene and the *E. coli sodA* gene have a common feature in their promoter region—both have a Fur box overlapping a perfect dyad symmetry (56). The significance of this observation remains unclear; further analyses of the Arc regulation system and of the regulation of *acn* expression in *L. pneumophila* are needed.

Using two different assays, we demonstrated that MICP is an aconitase. In the assay that measured the aconitase activity of MICP in solution, we observed an exponential increase in *cis*-aconitate formation, at least early in the reaction, rather than the linear increase that was expected and observed with the mitochondrial aconitase that was included as a positive control. It may be noteworthy that in this assay, the mitochondrial aconitase was "activated" according to the manufacturer's instructions whereas MICP was not subjected to any particular pretreatment. Reductant-based autoactivation of MICP during the assay may have accounted for the increase in its activity with time. Reduction is known to activate aconitase by reloading the $[\text{3Fe-4S}]$ cluster with an iron atom (34). In the assay used, the substrate (isocitrate) and the product of the enzymatic reaction (citrate) are reducing agents that could progressively reduce MICP and thereby activate it.

Comparison of MICP with the other sequenced aconitases showed that MICP (like *E. coli* aconitase) is more closely related to the cytosolic aconitase IRE-BP than to the mitochondrial aconitase. This suggests that MICP, like IRE-BP, could have another role apart from its enzymatic function in the Krebs cycle. Thus far, we have detected no IRE-specific RNA-binding activity in gel retardation assays with IRE structures based on the eucaryotic IRE sequences. We cannot exclude the possibility that MICP has a function analogous to that of IRE-BP, but we have yet to identify and test the bacterial IRE equivalent sequence.

That bacterial aconitase, mitochondrial aconitase, and IRE-BP have common regions of homology indicates that the three proteins evolved from a common ancestor. Whether mitochondrial aconitase evolved from bacterial aconitase or both evolved separately from a common ances-

FIG. 6. Multiple alignment for amino acid sequences of *L. pneumophila* MICP (*L. pneu* MICP), *E. coli* aconitase (*E. coli* ACN), human IRE-BP (Hum. IRE-BP), and porcine heart mitochondrial aconitase (Porc. mito.). The alignment of *E. coli* aconitase with human IRE-BP and porcine heart mitochondrial aconitase has been described previously (48). The amino acid sequence of human IRE-BP has been updated according to Hirling et al. (24). Insertions and deletions are indicated by dashes. Amino acids identical to those in MICP are indicated by a space. Active-site residues of the porcine heart mitochondrial aconitase (39) are indicated under the sequence as follows: ▲, residues involved in substrate recognition; ○, residues providing hydrogen bonds supporting active-site side chains; △, residue S642 and histidine-carboxylate pairs (D100-H101, D165-H147, E262-H167) implicated in catalysis; ◆, residues involved in Fe-S cluster ligation and interaction. The position at which an active-site residue is conserved in all sequences except MICP is boxed (K696).

FIG. 7. MICP exhibits aconitase activity. (A) Assay for aconitase activity of MICP in solution. Aconitase activity was measured spectrophotometrically at 240 nm by monitoring the formation of *cis*-aconitate from isocitrate. □, Control with no enzyme; ○, control with 4.8 µg of MICP and no substrate. (B) Assay for aconitase activity of MICP in a gel. MICP (10 µg) was subjected to electrophoresis under nondenaturing conditions in a 7.5% acrylamide gel and stained for aconitase activity as described in the text. OD, optical density.

tor remains open to speculation. The very high homology between IRE-BPs and bacterial aconitases suggests that IRE-BPs evolved from prokaryotes. That mitochondrial aconitase appears to lack IRE-BP activity and is shorter than IRE-BP suggests that it may have evolved to fulfill a more limited function in eukaryotic cell physiology.

MICP may have other roles apart from an IRE-BP-like activity. First, it could have a DNA-binding activity involved in the regulation of gene expression in response to cellular iron, as has been suggested for another iron-sulfur protein, ferredoxin I from *Azotobacter vinelandii* (58). Second, as MICP is highly expressed in *L. pneumophila* and contains 4 atoms of iron per molecule, it could play a role in iron storage. Third, MICP could play a role in defense against nitric oxide and toxic oxidative metabolites. Nitric oxide destroys Fe-S clusters (23). Hydrogen peroxide inactivates plant aconitase (59), and superoxide inactivates *E. coli* aconitase by inducing the loss of iron from the [4Fe-4S] cluster (15, 16). High concentrations of MICP might scavenge nitric oxide and oxidative radicals and divert them from other targets within the bacterial cell. However, several observations argue against these hypotheses for additional functions of MICP. Against the first hypothesis, regulatory proteins are usually not highly expressed, as is MICP. Against the second hypothesis, expression of iron storage proteins is typically iron regulated, and our evidence suggests that MICP is not. Against the third hypothesis, there is no convincing evidence that significant nitric oxide production occurs in human mononuclear phagocytes. Furthermore, phagocytosis of *L. pneumophila* in human monocytes is mediated through complement receptors that do not trigger an oxidative burst (46). Nevertheless, it remains possible that *L. pneumophila* is exposed to reactive nitrogen or oxygen intermediates in other environmental settings, e.g., inside amoebae.

The most striking feature distinguishing MICP from the other aconitases is an amino acid replacement in a position reported to be crucial for substrate binding and enzymatic

activity in a mutational analysis study of porcine heart mitochondrial aconitase (61). The substitution of a lysine for an arginine at position 580 of porcine mitochondrial aconitase reduced its enzymatic activity by 250- to 30,000-fold (61). If the presence of lysine at the homologous position (696) of MICP affects its aconitase activity at all, the impact must be far less profound. The specific activity of MICP measured in this study (15.8 U/mg) is only slightly less than that reported for *E. coli* aconitase (≥ 24.5 U/mg) (49) or mammalian aconitase (28 to 33.6 U/mg) (34). Such small differences in enzymatic activity among proteins must be interpreted with caution, since differences in the degree of protein purity, in the harshness of the purification process, and in the methods used for assaying enzymatic activity may influence these measurements. Nevertheless, the presence of a lysine at position 696 might render MICP relatively inefficient, and *L. pneumophila* might compensate for the relative inefficiency of the enzyme by expressing higher concentrations of it. The need to overproduce an enzyme containing 4 Fe atoms per molecule may contribute to the bacterium's high metabolic requirement for iron. To explore this hypothesis, we plan to study the effect of a K696R mutation in MICP on its enzymatic activity and on the iron requirement of *L. pneumophila*. If the mutation does in fact reduce aconitase activity, even partially, then the preservation through evolution of a mutation that reduces a crucial enzymatic activity would constitute another argument in favor of an additional role for MICP in bacterial physiology, one that confers a net evolutionary advantage to the organism.

The abundance of MICP suggests that *L. pneumophila* has a high metabolic requirement for aconitase and perhaps tricarboxylic acid cycle activity or that *L. pneumophila* requires large amounts of the protein for some other essential function. In any case, understanding the need for large amounts of this iron-rich protein could at least partly explain *L. pneumophila*'s high metabolic requirement for iron.

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