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Gene synthesis, bacterial expression and purification of the *Rickettsia prowazekii* ATP/ADP translocase

Mikhail F. Alexeyev, Herbert H. Winkler *

Laboratory of Molecular Biology, Department of Microbiology and Immunology, University of South Alabama College of Medicine, Mobile, AL 36688, USA

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Abstract

The *Rickettsia prowazekii* ATP/ADP translocase (Tlc) is the first member of a new family of ATP/ADP exchangers that includes both prokaryotic and eukaryotic proteins. We optimized the codon usage for expression of *tlc* in *Escherichia coli* by means of gene synthesis, expressed the synthetic gene in *E. coli*, and purified a modified Tlc that contained a C-terminal tag of 10 consecutive histidine residues by immobilized metal affinity chromatography. Although codon usage in *R. prowazekii* is very different from *E. coli*, the optimization of the codon usage by itself was insufficient to improve expression. However, the change of the cloning vector from pET11a to pT7-5 led to a 3–10-fold increase in the specific ATP transport rate by cells expressing the synthetic construct. The authenticity of the purified protein was confirmed by N-terminal amino acid sequencing and a matrix assisted laser desorption/ionization mass spectrometry.

Keywords: ATP; Membrane transport; Mitochondrium; Rickettsia; Gene synthesis; Expression

1. Introduction

The obligate intracellular bacterium *Rickettsia prowazekii*, the etiologic agent of epidemic typhus, grows and multiplies only within the cytoplasm of a eukaryotic cell. As an adaptation to this unusual ecological niche, *R. prowazekii* evolved to possess transport systems that allow it to take advantage of the many preformed metabolites that are abundant in the host cell [1–4]. Some of these transport systems have never been described in free-living bacteria. The ATP/ADP translocase (Tlc) that performs an exchange of ADP, generated as a result of rickettsial metabolism, for the high-energy ATP of host cell is an example of such system [4]. Although ancestral rickettsiae are the favorite candidate for the endosymbiote that led to mitochondria [5], and mitochondria possess a functionally similar system (AAC) that transports mitochondrial ATP in exchange for cytosolic ADP [6], the physiological direction of ATP transport in these two systems is opposite. That is, the mitochondrial transport system is 'altruistic' in the sense that it increases the abundance of ATP in the cytosol, whereas the rickettsial Tlc is 'selfish' in that it depletes the host cell's cytoplasm of ATP.

The physiological direction of ATP transport is not the only difference between Tlc and AAC. Tlc is insensitive [4,7] to carboxyatractyloside and bongkrekic acid, which are specific inhibitors of AAC [8–10]. The rickettsial ATP/ADP translocase

* Corresponding author. Fax: +1-334-460-7269;
E-mail: herbertw@sungcg.usouthal.edu

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gene (*tlc*) was cloned [11] and sequenced [12] and, based on the deduced amino acid sequence, the rickettsial protein is almost twice as large (56 822 Da) and shares no meaningful sequence homology with mitochondrial analogs. The topological model of Tlc generated by antibody accessibility [13] and the dual reporter approach [14] suggests that Tlc polypeptide crosses the cytoplasmic membrane 12 times, with its N- and C-termini facing the cytoplasm. The mitochondrial transporter is a functional homodimer and each monomer crosses the mitochondrial inner membrane six times. Therefore, the functional unit of the mitochondrial transporter, just like Tlc, has 12 transmembrane domains and almost the same molecular weight. Recently, a homologous ATP/ADP transporter was described in another obligate intracellular bacterium *Chlamydia trachomatis* [15,16] and, in addition, several members of this family were found in plant plastids [17–19]. Interestingly, all Tlc homologs share the same direction of ATP transport [20]. It is tempting to speculate that dissimilarities between non-mitochondrial homologs and mitochondrial ADP/ATP carriers are related to differences in the physiological direction of ATP transport. Clearly, studies on structure-function relationships in Tlc will be able either to lend support for or to disprove this hypothesis. However, to date these studies have been limited by the availability of Tlc in pure form and primary information on the protein per se.

Attempts at overexpression of Tlc in *E. coli* using the wild-type gene have met with only limited success [13,21]. Apart from the facts that membrane proteins often are very difficult to overexpress [22] and that Tlc seems to be toxic to *E. coli* [21], the low GC content of *R. prowazekii* (29%) may contribute to difficulties in expression through the usage of codons that are rarely encountered in *E. coli* genes [23]. In a related study, optimization of the codon usage for the dihydrofolate reductase of *Plasmodium falciparum*, which is also an organism with low GC content [24], allowed successful overexpression of the protein [25]. Here we report the construction of a synthetic *tlc* gene optimized for expression in *E. coli*, the expression of that gene, the purification of the His-tagged Tlc protein by immobilized metal affinity chromatography (IMAC), and the comparison of the N-terminal sequence and molecular mass of the purified protein with that deduced.

2. Materials and methods

2.1. Gene synthesis

Oligonucleotides for gene synthesis by both recursive PCR [26] and assembly of oligonucleotides representing only one of the strands [27] were designed to have 18–22 base overlaps. Unphosphorylated oligonucleotides were synthesized by Integrated DNA Technologies and used without further purification. The reactions contained 4 pmol of each gene synthesis oligonucleotide, 100 pmol of each PCR primer, 200 μM of each dNTP, 2 units of Vent DNA polymerase and 10 μl of 10×ThermoPol buffer (New England Biolabs) in total volume of 100 μl. The PCRs were carried out for 25 cycles (95°C for 1 min, 50°C for 1 min and 72°C for 1 min). DNA manipulations were performed by established methodology [28].

2.2. ATP transport assays

Cultures of *E. coli* were grown in Terrific Broth (TB [28]) and induced by addition of 1 mM IPTG. At different time points after induction, duplicate 1 ml portions of induced cultures were taken and the *E. coli* cells were sedimented by centrifugation at 5000×*g* for 5 min at room temperature. The cells were washed once with an equal volume of KPi buffer (50 mM potassium phosphate, pH 7.5) and resuspended in 500 μl of the same buffer. Transport was initiated by the addition of [α-^{32}P]ATP in KPi buffer to a final concentration of 50 μM and 1 μCi ml^{-1}. To determine the rate of transport, 100 μl aliquots were removed at various times and filtered on prewetted membrane filters (HAWP 02500, Millipore). The filters were washed once with KPi buffer, placed in the vial with 5 ml Ready-Safe scintillation cocktail (Beckman Instruments) and the incorporated radioactivity was measured by liquid scintillation spectrometry. The protein concentration of the samples was determined with the BC reagent kit (BioRad) according to the manufacturer's recommendations

and ATP transport rates were expressed as pmol ATP (mg protein)$^{-1}$ min^{-1}.

2.3. Cytoplasmic membrane preparations

E. coli cells were grown and induced as described above for ATP transport assays and collected by centrifugation at 5000×*g* for 15 min at 4°C. The cell pellet was resuspended in KPi buffer containing DNase and RNase (10 μg ml^{-1} each), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1× protease inhibitor cocktail (PIC, Boehringer) and 1 mM $MgCl_2$ at 0.1 mg wet weight ml^{-1} and disrupted by three passages through a French pressure cell at 20 000 lb in^{-2} (152 MPA). Unbroken cells and cell debris were removed by centrifugation at 10 000×*g* for 30 min at 4°C. The membranes were then sedimented by centrifugation at 150 000×*g* for 90 min at 4°C.

2.4. Purification of His-tagged ATP/ADP translocase

A membrane pellet derived from 500 ml of *E. coli* culture at OD_{600} = 2.3 was resuspended in 8 ml of buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM PMSF, 1× PIC) plus 20 mM imidazole and then solubilized by the addition of 20% *n*-dodecyl maltoside (DM) to a final concentration of 2%. Solubilization continued for 20 min on ice, after which insoluble material was removed by centrifugation at 150 000×*g* for 15 min at 4°C. The resulting supernatant fluid was incubated with 2 ml of Ni-NTA resin slurry (bed volume 1 ml, Qiagen) for 3 h at room temperature with constant mixing. The slurry was added to two 2 ml empty columns (Pierce), washed with buffer A containing 100 mM imidazole and 0.2% DM, and Tlc was eluted with buffer A containing 0.2% DM and 300 mM imidazole.

2.5. Western blotting, protein sequencing and mass spectroscopy

The purified Tlc preparation was separated by SDS-PAGE, transferred to PVDF membranes (ProBlott, Applied Biosystems) in a carbonate-bicarbonate buffer system [13], and subjected to seven cycles of Edman degradation at the core facility of the State University of New York at Brooklyn on a Procise Sequencer (Applied Biosystems). Molecular weight of the His-tagged Tlc was determined by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) at the Columbia University Protein Chemistry Core Facility using a PerSeptive Biosystems Voyager DE-RP mass spectrometer.

3. Results and discussion

3.1. Synthesis of the tlc gene

The synthetic *tlc* gene was designed to contain primarily those codons most frequently used by *E. coli* for highly expressed proteins. To aid in the assembly of the synthetic gene and to facilitate our ongoing site-directed mutagenesis and topology studies, unique restriction endonuclease sites were introduced in almost every region of the gene coding for a putative hydrophilic domain. The coding region of the gene was subdivided into three fragments, which were synthesized separately, cloned in pBluescriptII, sequenced and assembled into a full-size gene taking advantage of the restriction sites introduced during the gene design (Fig. 1). Two different strategies were employed during the synthesis of the *tlc* gene. The 5′ and 3′ portions of the gene were synthesized using the assembly of oligonucleotides representing only one of the strands [27], whereas the central portion of the gene was synthesized using the recursive PCR technique [26]. Since on several occasions we observed poor extension of primers longer than 65 bases by Vent DNA polymerase, the recursive PCR method was modified by introducing short PCR primers complementary to the 5′ and 3′ ends of the DNA fragment to be synthesized. No difference was found between the two methods in terms of fidelity of the gene synthesis. Of four independent clones sequenced for each product of a PCR reaction, only one of the N-terminal clones (395 bp) did not have any mistakes and all clones of the longer central and C-terminal fragments (566 and 568 bp, respectively) had one or two mistakes. However, since these mutations were located in different regions of the longer fragments, it was possible to assemble desired sequences by swapping unmutated portions using engineered restriction sites. The recursive PCR method is more economical in terms of the

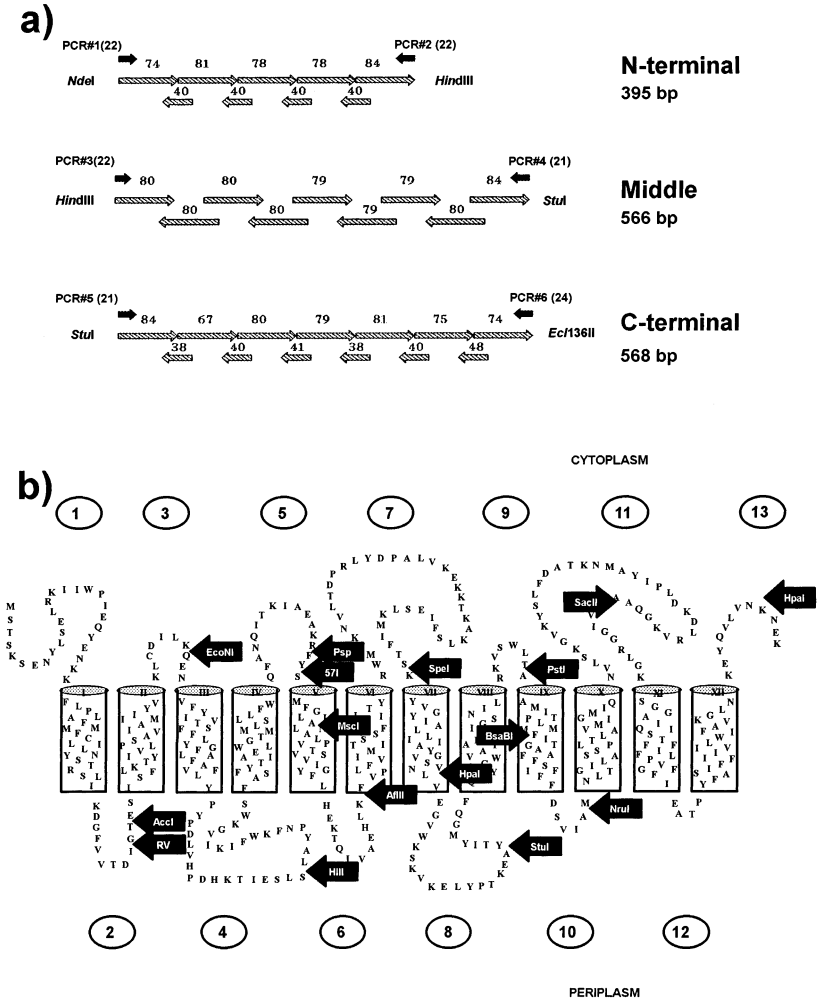

Fig. 1. Synthesis of the *tlc* gene. (a) Strategy. The synthetic gene was assembled from three fragments, which were synthesized separately. Oligonucleotides used for the synthesis are indicated by arrows, which point in 5′ to 3′ direction. Oligonucleotide length is shown above corresponding oligonucleotides. Short oligonucleotides used to amplify final products are designated as PCR with a number 1 through 6. Position is shown only for the relevant restriction sites. (b) A topological model of the Tlc. Positions of the restriction endonuclease cleavage sites are superimposed over amino acid sequence. Sites abbreviated as follows: AccI = *Acc*65I, RV = *Eco*RV, HIII = *Hin*dIII, Psp = *Psp*1406I, 57I = *Eco*57I.

total cost of oligonucleotides necessary to synthesize a DNA fragment of given length and, therefore, can be recommended as a method of choice for gene synthesis.

3.2. Expression of the synthetic tlc gene in E. coli

This synthetic *tlc* gene (GenBank AF087957) was modified in two ways: (i) a C-terminal tag consisting of 10 consecutive histidine residues preceded by the *Ecl*136II restriction site and followed by a translation termination codon was introduced by PCR using primers PCR#5 (5′)GAGGCCTACACCATCTACATG and (5′)GGGATCCTCAGTGGTGGTGGTGGTGGTGGTGGTGGAGCTCTTTTTCGTTTTTGTTAACCAGAACC, and (ii) the *Nde*I site at the 5′ end of the gene was replaced with *Bam*HI site by PCR using primers (5′) GGGATCCATGTCTACTTCCAAATCTGAAAAC and PCR#2 (5′) AGACAAGCTTTCGATAGTTTTG. The latter was done to facilitate the replacement of the *lac*Y gene in the expression vector pT7-5(*lac*Y) with the *tlc* gene. Two constructs, pMA605 and pMA606 (Fig. 2), were created as a result of the

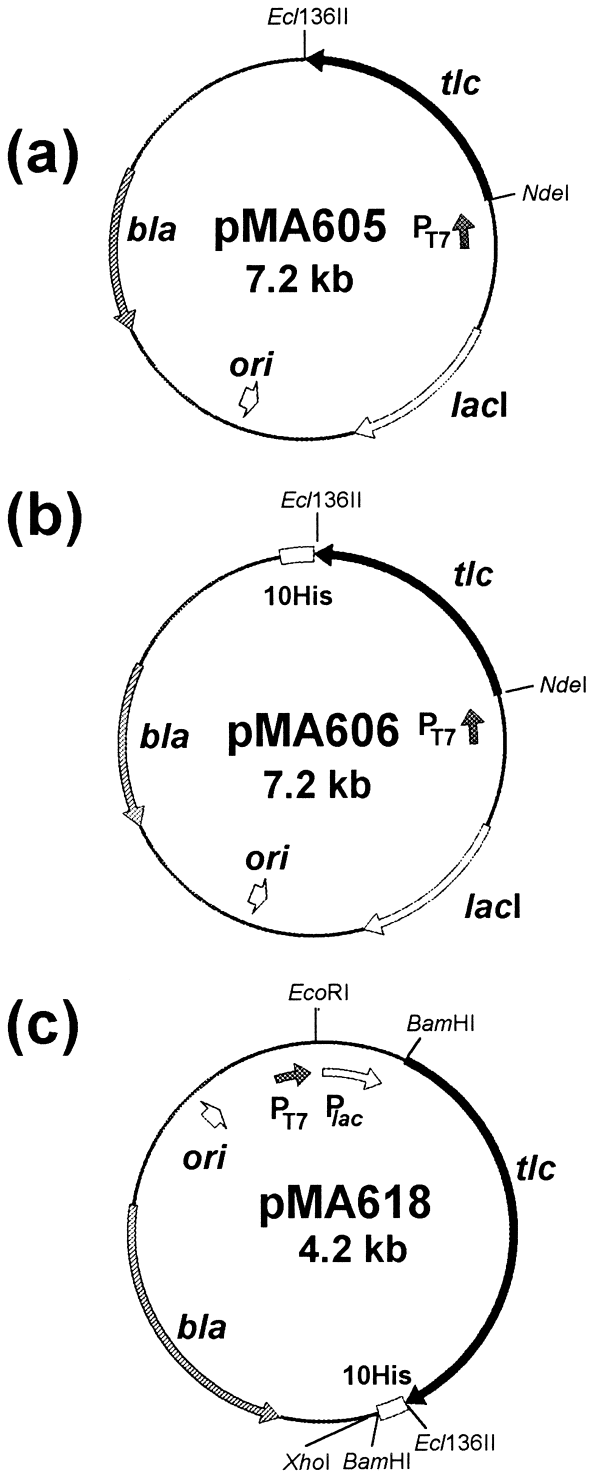

Fig. 2. Physical maps of the plasmids used in the study. Only restriction enzyme sites relevant for the present work are shown.

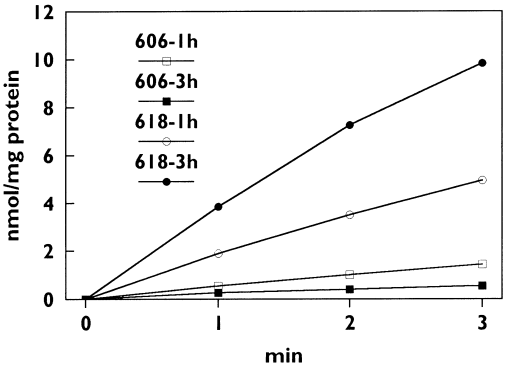

Fig. 3. Tlc expression directed by pMA606 and pMA618 in BL21(DE3) cells. Cultures were induced with 1 mM IPTG for 1 h (open symbols) or 3 h (solid symbols) and ATP uptake rates were measured. Results of a representative experiment are shown.

cloning of the original and His-tagged synthetic *tlc* genes, respectively, in the pET11a expression vector (Novagen), devoid of the *Pst*I site in the *bla* gene. The third expression construct (pMA618) was created by replacing the *lac*Y gene in pT7-5(*lac*Y) [29] with the His-tagged synthetic *tlc* gene (Fig. 2).

Both ATP uptake rates and total cellular levels of the Tlc protein, as judged by Western blotting (data not shown), were similar in BL21(DE3) cells expressing synthetic and wild-type *tlc* genes cloned in the pET11a vector. This suggests that either rare codons in the wild-type *tlc* gene are not the limiting factor for its expression in *E. coli*, or that the synthetic *tlc* gene has gained some new feature (e.g. unfavorable mRNA secondary structure) that counterweights the optimization of the *tlc* codon usage. Addition of the C-terminal His-tag to the Tlc protein destroyed the epitope for the antibody raised against a synthetic peptide, corresponding to the predicted 17 carboxy-terminal amino acids [13]. A similar phenomenon was also observed by others with Tet protein [30]. This made the comparison of total cellular levels of the Tlc expressed by synthetic and His-tagged synthetic *tlc* gene constructs impossible. However, there were no differences in the ATP uptake rates between *E. coli* cells expressing either synthetic or synthetic His-tagged *tlc* in pET11a vector (not shown), suggesting that C-terminal His-tag does not dramatically affect the activity of the membrane-inserted Tlc.

Since the optimization of the *tlc* codon usage did not result in an elevated level of protein synthesis, we were interested to see if changing the vector would

improve expression. Roepe et al. [29] reported the overexpression of another bacterial membrane protein, the lactose permease of *E. coli* (LacY), cloned in a different vector, pT7-5, in which the *lac*Y gene is under control of both the T7 and *lac* promoters. We replaced the *lac*Y gene in pT7-5(*lac*Y) with the His-tagged *tlc* gene creating pMA618 (Fig. 2). Upon the transformation of *E. coli* BL21(DE3) cells with pMA618 we observed both opaque and translucent colonies, a phenomenon similar to that described previously [31]. However, in contrast to previous observations, opaque colonies in our case grew better and had higher expression levels of Tlc as judged by ATP uptake rates (not shown). Therefore, only opaque colonies were used in further experiments. The BL21(DE3) cells bearing pMA618 had 3–10-fold higher ATP uptake rates than those bearing pMA606, the pET11a-based construct, suggesting higher expression levels (Fig. 3).

In BL21(DE3) the gene for T7 RNA polymerase is present in the chromosome under the control of the *lac* promoter-operator system. It has been demonstrated previously [31] that BL21(DE3) cells predisposed to form opaque colonies upon transformation with expression constructs have high basal levels of the T7 RNA polymerase. Indeed, introduction of pMA618 into BL21(DE3)[pLysS] cells, which express the T7 lysozyme gene, whose product has an inhibitory effect upon the activity of the T7 RNA polymerase, lead to the formation of uniformly translucent colonies. High basal expression is suggested by the observation that induction with IPTG of the *E. coli* BL21(DE3) derived from opaque colonies had

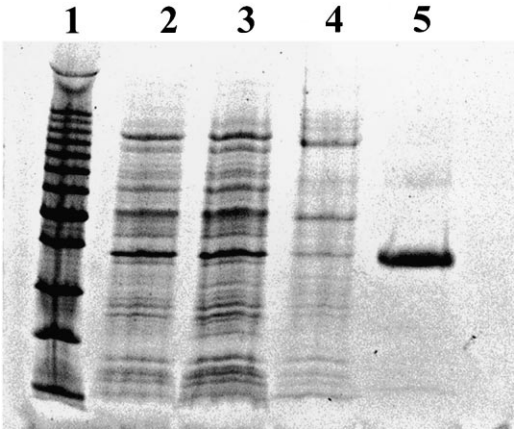

Fig. 4. Purification of Tlc by IMAC. Protein fractions from different stages of purification were subjected to SDS-PAGE and stained with Coomassie blue. Lane 1, 10 kDa protein marker (Gibco), the marker lanes are spaced by 10 kDa; lane 2, solubilized membranes; lane 3, flow-through; lane 4, wash; lane 5, purified Tlc, 38 kDa.

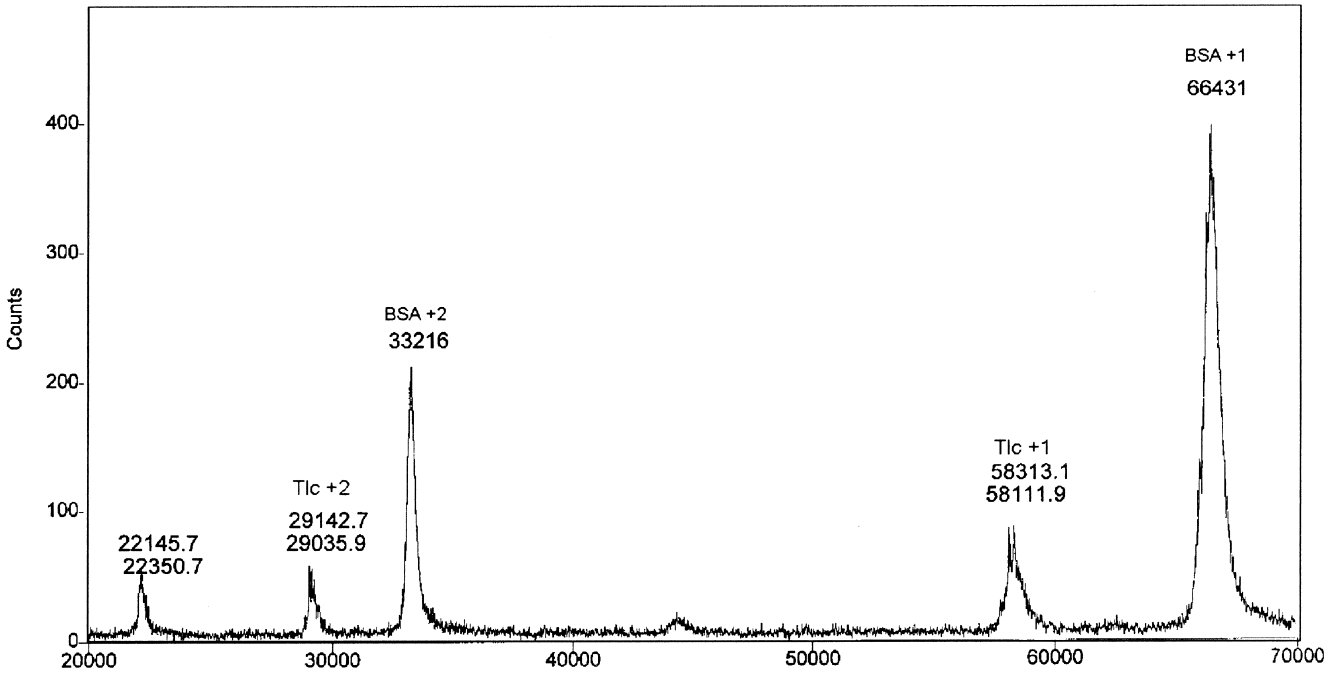

Fig. 5. MALDI-TOF spectrum of the purified His-tagged Tlc. BSA, bovine serum albumin was used as internal standard.

only marginal effect upon Tlc expression. Therefore, it appears that, counter intuitively, the tight repression of *tlc* expression observed in the pET11a vector is not advantageous for obtaining the highest levels of active ATP/ADP translocase.

3.3. Purification and characterization of the His-tagged Tlc

The His-tagged Tlc (>90% pure) was purified with a yield of about 1 mg l^{-1} of bacterial culture as estimated by SDS-PAGE. It was found that utilization of DM throughout the purification protocol results in more pure protein preparations as compared to the identical protocol employing *n*-lauroyl sarcosine (not shown). The purified protein migrates in SDS-PAGE with an apparent molecular weight of 38 kDa (Fig. 4), which is substantially lower than the molecular weight derived from its predicted amino acid sequence (58 435 Da). This could be due to excessive binding of SDS to hydrophobic regions of protein, a phenomenon that has been commonly suggested for many membrane proteins including Tlc [13]. Alternatively, a downstream methionine, rather than methionine assumed to be the start methionine may be the initiation codon in *R. prowazekii*. In addition, proteolytic processing may have removed the N-terminal portion of the protein. Finally, since BL21(DE3) cells possess a major protein with apparent molecular weight of 38 kDa and others [30] have observed in this strain an endogenous host protein which had a similar molecular weight, was induced with IPTG and had an affinity for the Ni-column. For all these reasons it was important to confirm the identity of the purified protein. Therefore, N-terminal amino acid sequencing was performed on membrane-immobilized SDS-PAGE and IMAC purified protein. The first seven N-terminal amino acids STSKSENY exactly matched the predicted sequence of Tlc protein, except that the initiating methionine residue had been removed by the *E. coli* methionine aminopeptidase. The MALDI-TOF spectrum of the purified His-tagged Tlc preparation had two maximums at 58 112 Da and 58 313 Da (Fig. 5), both in very good agreement with the molecular weight calculated on the basis of the amino acid composition (58 305 Da).

The results of this study can be enumerated as follows:

1. The codon usage for the *tlc* gene was optimized for the expression in *E. coli* by gene synthesis.
2. Optimization of the codon usage did not lead to the elevated expression of the synthetic gene cloned in the pET11a as compared to the wild-type gene. This suggests that either rare codons in the wild-type *tlc* gene are not the limiting factor for its expression in *E. coli*, or that the synthetic *tlc* gene has gained some new feature(s) (e.g. unfavorable mRNA secondary structure) that counterweight the optimization of the *tlc* codon usage.
3. The change of the cloning vector to pT7-5 resulted in the 3–10-fold increase in the expression as judged by the ATP uptake rates.
4. Preparations of >90% pure His-tagged Tlc were obtained and protein authenticity was confirmed by N-terminal sequencing and mass spectrometry.
5. Recombinant Tlc expressed in *E. coli* is processed by the methionine aminopeptidase to remove initiating formylmethionine.
6. The discrepancy between the molecular weights of Tlc predicted from the amino acid sequence and apparent from SDS-PAGE is not due to the proteolytic modification.

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