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Heterologous Expression of the Subunits of the Na^+ V-ATPase of the Thermophile *Caloramator fervidus*

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ABSTRACT

The V-type ATPase of the thermophile *Caloramator fervidus* is an ATP-driven Na^+ pump. The 9 genes in the *ntpFIKECGABD* operon coding for the 9 subunits of the enzyme complex were expressed separately in *Escherichia coli*. Except for subunit G, all subunits were produced. The main V_1 subunits A and B and central stalk subunit C were produced as soluble proteins, while the main V_0 subunits I and K ended up in the membrane fraction following cell fractionation. Stalk subunit E had a strong tendency to aggregate and was distributed over the soluble and membrane fractions, while subunit D was produced in inclusion bodies. Expression of subunits D and E in the Gram positive host *Lactococcus lactis* reduced aggregation and breakdown significantly, but subunit D was still produced in inclusion bodies.

Keywords: central stalk, inclusion bodies, molecular motor, Na^+ pump, V_0V_1-ATPase

INTRODUCTION

Caloramator fervidus is a strictly anaerobic bacterium that was isolated from a hot spring in New Zealand. The thermophilic organism grows optimally at 68°C (Patel *et al.* 1987) on amino acids and peptides that are taken up into the cell by transport systems that exclusively are coupled to Na^+ (Speelmans *et al.* 1993a, 1993b). The organism is unique in that it does not support a proton cycle; it does not maintain an electrochemical proton gradient across the cell membrane, but, instead, a large electrochemical gradient of sodium ions is generated (Speelmans *et al*. 1994). The Na^+ gradient is maintained by a V-type ATPase, a type of ATPase that is only rarely observed in the bacterial kingdom (Lolkema *et al.* 2003). Recently, we presented the sequence of the *ntpFIKECGABD* operon that codes for the Na^+ V-type ATPase of *C. fervidus* (Ubbink-Kok *et al*. 2006).

V-type ATPases are commonly found in the endomembranes of eukaryotic cells where they serve diverse physiological functions (for a review, see Nelson 2003). They are rotary motors similar to F-type ATPases from bacteria and eukaryotes, but differ structurally in the way the two motors are connected, a feature they share with A-type ATPases found in archaea (for reviews see Stock *et al*. 2000; Müller and Grüber 2003). Sequence analysis of the subunits strongly suggests that the eukaryotic V-type ATPases originate from the archaeal A-type ATPases (Hilario and Gogarten 1998; Lolkema *et al*. 2003). V-type ATPases in the bacterial kingdom are exceptional and sequence analysis shows that they are most closely related to the archaeal A-type ATPases, suggesting they have crossed the barrier between archaea and bacteria by horizontal gene transfer. They are most abundantly found among the Gram positives with low CG content in the phylum Firmicutes. The presence and absence of the structural genes in species of the same genus supports a late acquisition. For instance, while the *Streptococcus* species *pyogenus* and *pneumoniae* contain the ntp operon on the chromosome, the species *mutants*, *agalactiae* and *thermophilus* do not. Similarly, the *Clostridia* species *tetani* and *perfringens* have a V-type ATPase, while *acetobutylicum* does not. Within the bacteria that contain a V-type ATPases a number of groups can be discriminated based on sequence relationships and operon organization that probably reflect multiple transfers between the archaeal and bacterial worlds (Lolkema *et al*. 2003). The gene order of the *ntpFIKECGABD* operon of *C. fervidus* is found in a group of bacteria in the phyla Firmicutes, Fusobacteria and Thermus/Deinococcus and corresponds to the gene order that is most frequently observed for the A-type ATPases of the Euryarchaeota. The phylogenetic relationship between the V-type ATPases in this group of bacteria which is similar for all subunits is depicted in **Fig. 1**. The *Caloramator* enzyme complex clusters with the two *Clostridium* enzyme complexes on one branch of the tree. The cluster is more closely related to the complex in *Fusobacterium nucleatum* in the phylum Fusobacteria than to the complexes in the other Firmicutes.

The V-type ATPase of *C. fervidus* pumps Na^+ ions out of the cell against the sodium ion motive force at the expense of the hydrolysis of ATP. The enzyme complex consists of an integral membrane part V_0 that is responsible for the translocation of the Na^+ ions and a cytoplasmic, membrane associated part V_1 that hydrolyzes ATP. V_0 is built of subunits K and I, V_1 consists of 7 subunits, termed A, B, C, D, E, F, and G (Höner zu Bentrup *et al*. 1997; Ubbink-Kok *et al*. 2000). ATP hydrolysis in V_1 drives the physical rotation of a stalk that is contained in the central cavity of a hexagonal arrangement of alternating A and B subunits. The central stalk is connected to a multimeric assembly of K subunits in V_0 (the 'rotor') that rotates in the plane of the membrane against the static part of V_0 formed by the I subunit. The rotation free energy is used to pump Na^+ ions across the membrane at the interface of the rotor and the I subunit. The connection between the two motors consists of two parts. The first, a central stalk that transmits the energy between the two by rotation and the second, a static part formed by peripheral stalks (the 'stator') that connect the I subunit in V_0 to the AB complex in V_1 thereby preventing energy dissipation by idle rotation.

In the past we have studied the V-type ATPase of *C. fervidus* by electron microscopy of the purified enzyme complex (Höner zu Bentrup *et al*. 1997; Ubbink-Kok *et al*.

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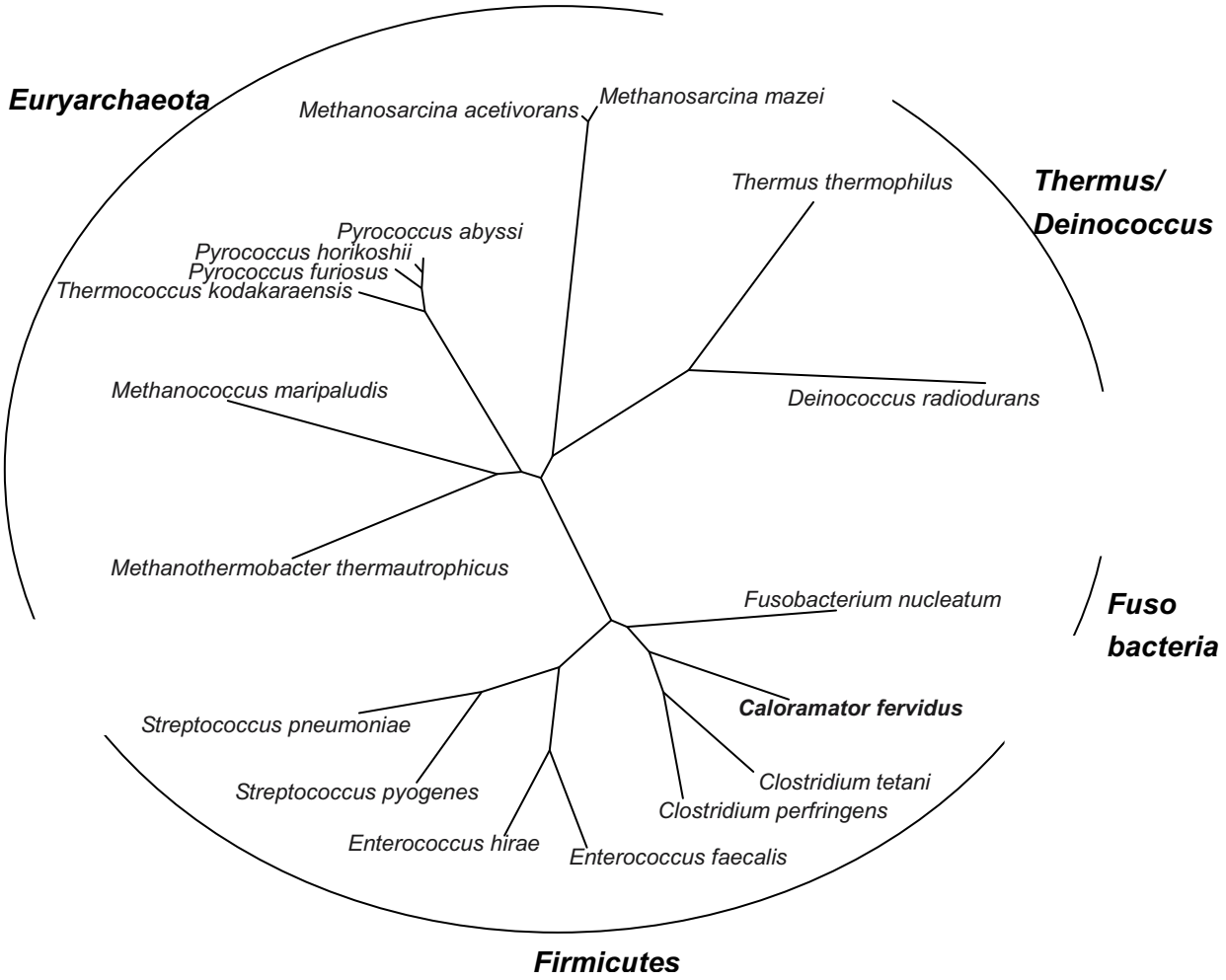

Fig. 1 Phylogenetic distribution of the *ntp* operon in bacteria and archaea. The tree is based on the NtpB subunit of the V-type ATPases found in the organisms indicated on the tree. A multiple sequence alignment was computed using ClustalX (Jeanmougin *et al*. 1998). The tree was constructed using the DrawTree program in the Phylip package (Felsenstein 1993).

2000) and showed that the 'stator' structure consisted of two peripheral stalks (Boekema *et al*. 1997, 1998, 1999; Ubbink-Kok *et al*. 2000) and that the central stalk was composed of two subunits (Chaban *et al*. 2002). A major challenge remains in the localization of the different subunits in the stalk regions. We plan to identify the constituents of the stalks by building subcomplexes from isolated subunits. Here, we describe the first step in this approach: the expression of the subunits of the *C. fervidus* complex in *Escherichia coli* and in *Lactococcus lactis*.

MATERIALS AND METHODS

Growth conditions

Caloramator fervidus ATCC 43024 was grown anaerobically at 68°C in trypton-yeast extract-glucose medium (Patel *et al*. 1987) as described (Ubbink-Kok *et al*. 2000). *Escherichia coli* DH5α was grown in Luria Bertani Broth (LB) medium at 37°C under continuous shaking at 150 rpm. When appropriate, ampicillin was added to the medium at a final concentration of 50 μg/mL. *E. coli* harboring the pBAD-HN plasmids carrying the genes coding for the different subunits of the V-type ATPase (see below) were grown until the optical density reached 0.6 measured at 600 nm followed by induction with 0.075% (w/v) arabinose. *Lactococcus lactis* NZ9000 was grown at 30°C in half-strength M17 broth (Terzaghi *et al*. 1975) containing 0.5% glucose and 5 μg/mL chloramphenicol when appropriate. *L. lactis* harboring plasmid pNZntpD or pNZntpE was grown until the optical density reached 0.6 measured at 600 nm followed by induction with 5 ng/mL nisin (Sigma-Aldrich, Steinheim, Germany) unless stated otherwise.

Plasmid construction

The sequence of the *ntp* operon of *C. fervidus* is deposited in GenBank under accession number DQ369724. The genes encoding the subunits of the V-ATPase were amplified by PCR using genomic DNA of *C. fervidus* as the template. The forward primer introduced a *Nco*I site around the initiation codon of the genes, the reverse primer a *Xba*I site downstream of the stop codons. The PCR products were digested with the two restriction enzymes and ligated into the corresponding restriction sites of vector pBADHN for expression in *E. coli* under control of the arabinose promoter. Vector pBADHN was constructed by ligating a fragment of double stranded DNA into the *Nco*I site of pBAD24 (Invitrogen). The fragment with *Nco*I site overhang at both ends coded for 6 histidine residues (His-tag) and was obtained by annealing two complementary polynucleotides. The sequence was such that following ligation, the *Nco*I site at the 5′ end of the His-tag was destroyed while the site at the 3′ end was left intact. The histidine codons were in frame with the upstream initiation codon and the downstream ATG codon in the *Nco*I site. The genes coding for subunits D and E were also cloned in vector pNZ8048 (Kunji *et al*. 2003) for expression in *L. lactis*. The resulting plasmids, named pBADHNntpX in which X stands for A, B, C, D, E, F, G, I, and K, and pNZntpD and pNZntpE code for the subunits extended with six (pBADHNntpX) or ten (pNZntpX) histidine residues at the N-terminus. The sequence of the inserts was confirmed (ServiceXS, The Netherlands) and, subsequently, the plasmids were introduced into *E. coli* DH5α and *L. lactis* NZ9000. The latter allows for expression under the control of the tightly regulated nisA promoter (Kuipers *et al.* 1993; de Ruyter *et al.* 1996).

Expression studies

Cells of a 400 mL culture of *E. coli* DH5α or *L. lactis* NZ9000 expressing the V-ATPase subunits were harvested by centrifugation and washed once with 50 mM KPi pH 7 at 4°C. The cells were resuspended in the same buffer containing 0.1 mM phenylmethylsulfonylfluoride at an optical density of ~50 measured at 600 nm. Subsequently, the cells were broken using a probe-sonicator operated at an amplitude of 6 microns while intermittently cooling on ice. Unbroken cells and debris were removed by centrifugation for 5 min at 6,000 rpm in a table top centrifuge (Eppendorf) at 4°C. The pellet was resuspended in the 50 mM KPi pH 7 buffer to the same volume as the original cell suspension (the 'low spin' pellet p1). The supernatant (crude cell extract) was further fractionated in a membrane fraction and a cytoplasmic fraction by ultracentrifugation in a Beckman tabletop centrifuge operated at 80,000 rpm for 30 min at 4°C. Again, the pellet was resuspended in the original volume of the cell suspension in the KPi buffer (the 'high spin' pellet p2). Alternatively, the crude cell extract was centrifuged for 30 min at 13,000 rpm in a Eppendorf table top centrifuge at 4°C. The latter procedure did not result in a complete separation between cytoplasmic and membrane fractions.

Cells from a 200 mL culture of *L. lactis* were resuspended in 1 mL of 50 mM tris pH 7 containing 0.5 M NaCl, 0.1 mM phenylmethylsulfonylfluoride and 1 mg/mL lysozyme. The suspension was incubated for 60 min at 30°C, cooled down on ice and, subsequently, the cells were disrupted by two passages through a French Pressure cell in the presence of 1 µg/mL of DNAase. The suspension was centrifuged for 10 min at 13,000 rpm in a Eppendorf table top centrifuge at 4°C yielding a pellet and supernatant fraction.

Samples of the pellet and supernatant fractions were loaded onto 12% or 14% SDS-PAGE gels and the proteins were transferred to a polyvinylidene difluoride membrane (Roche) by semidry electro blotting. His-tagged proteins were detected using a primary anti-his antibody (Amersham BioSciences) followed by chemiluminescent detection with CDP-Star (Roche) on a LumiImager (Roche).

Materials

All reagents were reagent grade and obtained from commercial sources.

RESULTS

Heterologous expression of the V-ATPase subunits in *E. coli*

The genes coding for the subunits of the *C. fervidus* V-type ATPase were cloned into vector pBAD-HN resulting in an open reading frame coding for the V-ATPase subunits extended at the N-terminus with 6 histidine residues (see Methods). Expression of the genes was under control of the arabinose promoter. *E. coli* DH5α cells expressing the different subunits were fractionated into a 'low spin' pellet (p1) containing unbroken cells and debris, a 'high spin' pellet (p2) containing cell membranes, and a cytoplasmic fraction (s) as described (**Fig. 2**; see Methods). Pellet fractions were resuspended in the original volume of the cell suspension and aliquots of the fractions were loaded onto a SDS-PAGE gel. The proteins were blotted and his-tagged proteins were visualized using an antibody raised against the His-tag. For all subunits except subunit G, the blots showed the production of his-tagged proteins with the expected molecular masses when the cells were grown in the presence of the inducer arabinose, while no bands were observed in its absence (see **Fig. 2A** for subunits I, A, B, E, and D; uninduced cells were not shown for subunits C, K, F, and G). V_0-subunit I showed up in the two pellet fractions, which is to be expected for a protein residing in the cytoplasmic membrane. V_1-subunits A and B that together form the headpiece of the V-ATPase were predominantly found in the cytoplasmic fraction, but some of the protein was detected in the high spin pellet, suggesting that both subunits are not fully stable and precipitated to some extent during the procedure. Stalk subunit E was distributed over the 'high spin' pellet and cytoplasmic fractions. In both frac-

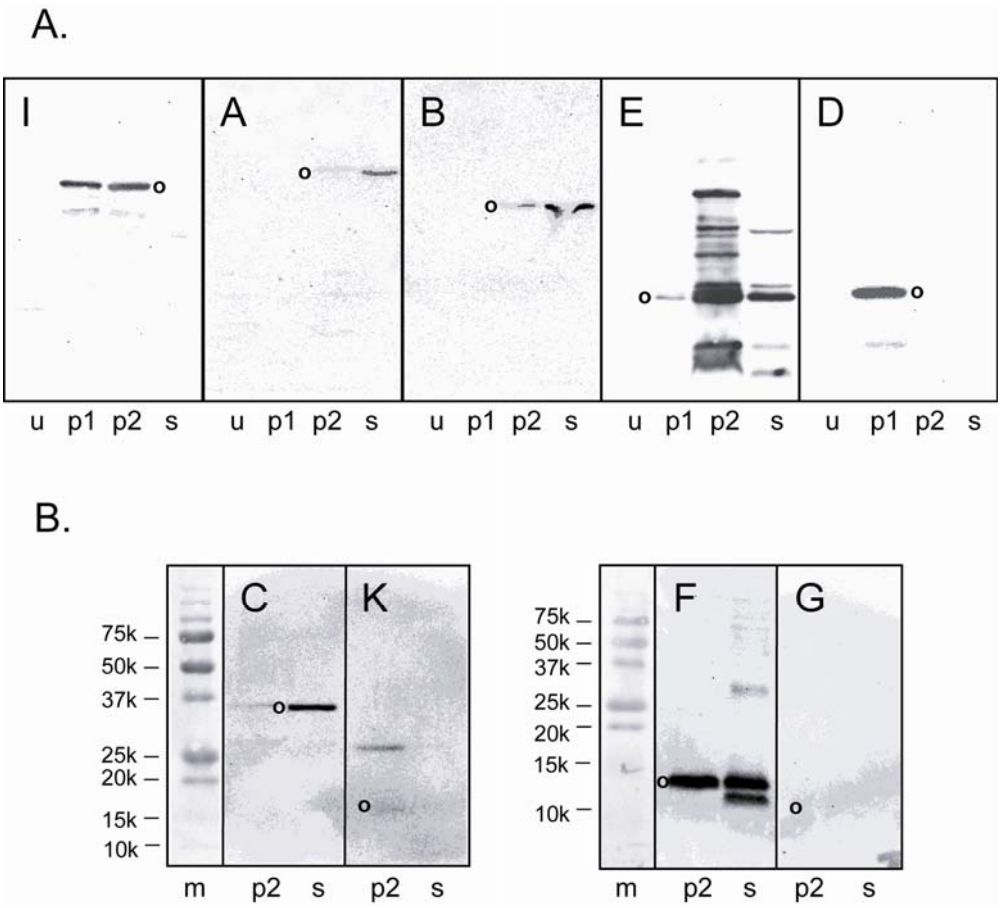

Fig. 2 Expression of the subunits of the V-type ATPase of *C. fervidus* in *E. coli*. (**A**) Subunits I, A, B, E, and D. Lane 'u', crude cell extract of uninduced cells, 'p1', low spin pellet of induced cells, 'p2', high spin pellet of induced cells, and 's', supernatant of induced cells. (**B**) Subunits C, K, F, G. Lanes 'p2' and 's', high spin pellet and supernatant of induced cells, respectively. Samples were loaded on a 12% (A, B left panel) or a 14% (B right panel) SDS-polyacrylamide gel, followed by electroblotting and detection of His-tagged proteins using antibodies targeted at the His-tag. See Methods for details. Lane 'm' shows the prestained marker proteins. The dot marks the expected position of the subunit based on the prestained marker set.

tions, bands with molecular masses higher and lower than the expected mass were observed suggestion both aggregation and degradation of the produced protein. Since only very little his-tagged protein was observed in the 'low spin' pellet (p1), it is concluded that subunit E is produced in *E. coli* not in inclusion bodies, but as a soluble protein with a strong tendency to aggregate and precipitate. The aggregates are spun down in the 'high spin' pellet. Stalk subunit D was produced in large amounts, but ended up quantitatively in the 'low spin' pellet, strongly indicating that it was produced in the cell as inclusion bodies. In contrast, the central stalk subunit C behaved like the soluble subunits A and B and was predominantly found in the supernatant fraction with a minor amount in the pellet fraction (**Fig. 2B**). The proteolipid subunit K isolated as part of the holocomplex runs with a molecular mass of 17 kDa (Ubbink-Kok *et al*. 2000), but most of the recombinant protein runs as a protein with a considerably higher mass. Nevertheless, a small fraction of the protein could be identified running at the expected position. Most likely, the recombinant protein forms dimers under the conditions of the electrophoresis. The small stalk subunit F was found both in the membrane and supernatant fractions. The part of the protein in the supernatant showed some tendency to aggregate as evidenced by the presence of protein bands with a higher molecular mass and a degradation product is visible as well. Subunit G, the smallest subunit of the V-ATPase complex was the only subunit for which no expression was observed in spite of several attempts.

Heterologous expression of subunits D and E in *L. lactis*

Expression of subunits D and E was studied in *L. lactis* using the NICE expression system (de Ruyter *et al.* 1996; Kunji *et al*. 2003). Cells harbouring plasmids pNZntpD and pNZntpE containing the *ntpD* and *ntpE* genes were grown in the presence of different amounts of the inducer nisin and the cells were disrupted in a French Pressure cell. The broken cell suspensions were separated in a low spin pellet and a supernatant fraction containing cytoplasmic membranes and the cytoplasmic fraction. A low spin to remove the unbroken cells and debris was omitted to ensure that all produced protein was recovered. Both plasmids produced proteins of the expected sizes in the presence of the inducer nisin and not in its absence (**Fig. 3**).

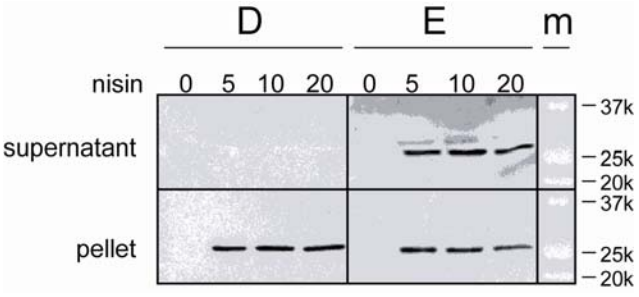

Fig. 3 Expression of subunits D and E in *L. lactis*. Cells were induced with the indicated amount of nisin and separated in a pellet and supernatant fraction as described in the Methods section. The samples were run on a 12% SDS-PAGE gel, followed by electroblotting and detection with antibodies raised against a His-tag.

Only small differences were observed with the different inducer concentrations. As in in *E. coli*, subunit D showed up exclusively in the low spin pellet, suggesting the formation of inclusion bodies in *L. lactis*. Subunit E was more or less equally distributed over pellet and supernatant fractions. Even though the centrifugation conditions were not exactly identical, the fraction of subunit E ending up in the low spin pellet appears to be higher in *L. lactis* than in *E. coli* (compare **Fig. 2A** and **Fig. 3**). A remarkable difference was the lack of aggregation observed upon expression in *L. lactis*, suggesting that the state of the protein is different when expressed in the two expression systems. To obtain further information about the state of the two proteins following expression in *L. lactis*, crude cell extracts prepared from cells expressing the two subunits (**Fig. 4**, lane 1) were incubated with 1% of the detergent Triton X100 to solubilize membranes and protein aggregates. Following centrifugation, the supernatant contained no detectable subunit D, but a significant amount of subunit E representing the soluble fraction (lanes 2). The pellets were washed twice by centrifugation using buffer containing 0.1% Triton X100. No His-tagged protein could be detected in the supernatants of the washing steps. The pellets were then resuspended in 6 M of the denaturant guanidiniumHCl followed by centrifugation. Essentially all of subunit D was recovered in the supernatant (lanes 5 and 6), while a very small amount of subunit E showed up in the supernatant. For both subunits, the original signal observed in the crude cell extract (lane 1) was apparently not recovered in the fractionation steps (lanes 2-6).

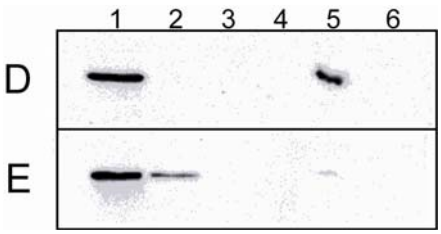

Fig. 4 Subunit D forms inclusion bodies in *L. lactis*. Cells were induced by 10 ng/mL of nisin. The crude cell extract (lanes 1) was treated with 1% Triton X100 for 30 min at 30°C. The suspension was centrifuged for 30 min in a Sorvall SS34 rotor operated at 18,500 rpm. The supernatant (lanes 2) was separated from the pellet and the latter was washed twice with 50 mM KPi buffer pH 6 containing 0.1% Triton X100 by centrifugation (lanes 3 and 4, supernatants). Finally, the pellet was resuspended in 6 M guanidinium and the suspension centrifuged for 30 min in a Beckman TL100.2 rotor operated at 53,000 rpm. The supernatant and pellet were loaded in slots 5 and 6, respectively.

DISCUSSION

Electron microscopy studies of subcomplexes is a potent approach in identifying the different subunits in the V-type ATPase complex. The subcomplexes may be isolated from the holoenzyme following dissociation by some means or by building the subcomplex from the separate subunits. Controlled thermal dissociation of the purified V-type ATPases of *C. fervidus* allowed for the isolation of two subcomplexes containing both the major V_1 subunits A and B. One of the two contained, in addition, the stalk subunits E and C, the other just subunit E. The structure of the two complexes was analyzed by electron microscopy and single particle analysis (Chaban *et al*. 2002). Difference mapping of the projections revealed that subunits E and C together formed the central stalk of the complex. Subunit E showed up as an elongated density, partly sitting in the central cavity of the AB complex in very much the same way as the γ subunit in the F-type ATPases, and partly sticking out. Subunit C showed up as a spherical density positioned at the tip of the E subunit providing the connection with the membrane embedded part. Subunit C has no counterpart in F-type ATPases where the γ subunit connects directly to F_0. A disadvantage of the latter approach is that the composition of the subcomplex cannot be controlled. This may be overcome in the second approach in which the subcomplexes are built from the isolated subunits, an approach that, for instance, was explored for the F-type ATPases (e.g. Miroux and Walker 1996; Arechaga *et al*. 2000, 2002). A prerequisite is that the set of subunits is available as separate entities. Then, in principle, the interaction between all combinations of subunits may be studied. With the availability of the structural genes, the

subcomplexes may be formed by mixing the subunits following expression and purification, or by expressing combinations of the genes in synthetic operons. A combination of both may be especially productive. The disadvantage may be that it may not be possible to obtain the subunits separately in their native conformation. Here, we show that except for subunit G, all subunits of the V-type ATPase of *C. fervidus* engineered with a N-terminal his-tag for purification purposes were produced upon expression in *E. coli* when expressed separately (**Fig. 2**). Moreover, subunits A, B, C, K and I ended up in the expected fractions after fractionation of the cells and, possibly, are in their native conformation. Subunits A and B form the major part of V_1 and their stability may be improved by expressing them in tandem. Expression of V_0 subunit I is remarkable since expression of the corresponding subunit of the F_1F_0-ATPase subunit of *E. coli* was not successful (Arechaga *et al*. 2003). Separate expression of subunits D and E in *E. coli* results in aggregation and breakdown for the former and inclusion bodies for the latter. Changing the host to the Gram positive lactic acid bacterium *L. lactis* resulted in an improved expression of subunit E (**Fig. 4**). A significant fraction of the protein was recovered in a soluble form that appeared to be much more homogenous than upon expression in *E. coli*. Expression of subunit D in *L. lactis* resulted in precipitates suggesting that also in this expression system inclusion bodies may be formed (Kunji *et al*. 2003). Alternatively, subunit D is not soluble in the water phase or it strongly associates with other insoluble cellular constituents.

The successful expression of subunits A, B, C, K, I and E allows for the next step in the procedure to determine the localization of the different subunits in the stalk regions of the ATPase complex by building subcomplexes from isolated subunits. The next step involves the purification of the subunits by Ni^{2+}-NTA affinity chromatography and the reconstitution of subcomplexes. Crucial to the approach will be the reconstitution of the V_1 headpiece consisting of three of the subunits A and B each. Successful formation of the subcomplexes will be followed by electron microscopy and biochemical techniques.

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