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The ATPase Activity of The *G2alt* Gene Encoding an Aluminium Tolerance Protein from *Anoxybacillus gonensis* G2

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The G2ALT gene was cloned and sequenced from the thermophilic bacterium *Anoxybacillus gonensis* G2. The gene is 666 bp long and encodes a protein 221 amino acids in length. The gene was overexpressed in *E. coli* and purified to homogeneity and biochemically characterized. The enzyme has a molecular mass of 24.5 kDa and it could be classified as a member of the family of bacterial aluminium resistance proteins based on homology searches. When this fragment was expressed in *E. coli*, it endowed *E. coli* with Al tolerance to 500 μM. The purified G2ALT protein is active at a broad pH range (pH 4.0-10.0) and temperature range (25°C-80°C) with optima of 6.0 and the apparent optimal temperature of 73°C respectively. Under optimal conditions, G2ALT exhibited a low ATPase activity with K_m- and V_{max}- values of 10±0.55 μM and 26.81±0.13 mg Pi released/min/mg enzyme, respectively. The ATPase activity of G2ALT requires Mg^{2+} and Na^{+} ions, while Zn^{2+} and Al^{3+} stimulate the activity. Cd^{2+} and Ag^{+} reduced the activity and Li^{+}, Cu^{2+}, and Co^{2+} inhibited the activity. Known inhibitors of most ATPases, like such as β-mercaptoethanol and ouabain, also inhibited the activity of the G2ALT. These biochemical characterizations suggested that G2ALT belongs to the PP-loop ATPase superfamily and it can be responsible for aluminium tolerance in *A. gonensis* G2.

***Keywords*:** aluminium tolerance gene (*G2alt*), aluminium tolerance protein (G2ALT), ATPase, cloning, aluminium tolerance, *Anoxybacillus gonensis* G2

Aluminium (Al) is a widespread and the third most abundant element after oxygen and silicon (8% of the earth's crust) (Fischer *et al*., 2002). Aluminium is insoluble at neutral pH but becomes soluble at a pH lower than 5.0 and has a toxic effect on most microorganisms and plants at higher concentrations (Kawai *et al*., 2000).

Most microorganisms and plants do not possess a tolerance mechanism towards stress conditions. When bacterial cells are exposed to potentially lethal concentrations of metals, they have to express a tolerance mechanism for their continued survival. Some bacteria and higher microorganisms have developed resistance to toxic metals and are able to make them innocuous. They respond to heavy metals using various defense systems, such as exclusion (Ortiz *et al*., 1992), compartmentalization (Valls *et al*., 2000; Klaus-Joerger *et al*., 2001), complex formation (Wang *et al*., 1997) and synthesis of metal-binding proteins, such as metallothioneins (Adamis *et al*., 2004). Some plants appear to use a simple mechanism of chelating Al using organic acids (Schildknecht and Vidal, 2002). Apart from these mechanisms, the membrane may also play an important role in metal homeostasis, either by preventing or reducing their entry into the cell or through efflux mechanisms. In bacteria, most resistance systems are based on the energy-dependent efflux of toxic ions (Silver, 1996; Silver and Phung, 1996). Seven major types of efflux are known, two of them being ATPases, (1) P-type ATPases, generally single polypeptide determinants and (2) ABC ATPases, ATP binding cassettes consisting of a cytoplasmic membrane-associated ATPase subunit and one or two membrane embedded pump channel subunits (Silver and Phung, 1996).

The molecular studies on aluminium tolerance in bacteria are limited. However, there are numerous studies on aluminium resistance in several plants (Sugimoto *et al*., 2004). Generally, these studies were conducted at the molecular level, for example, cloning the genes induced by Al. There are just a few studies on Al tolerance mechanisms in Al-tolerant microorganisms. The acidophilic bacterium, *Arthrobacter viscosus* ALRJ6 has a gene responsible for the aluminium resistance (Jo *et al*., 1997). The cloned fragment of this gene has been designated as *alu1-p* but the full sequence of the gene is still unknown. In addition, six Al-tolerant acidophilic microorganisms that possess a high level of resistance to Al (up to 300 mM) were characterized (Kawai *et al*., 2000).

The relationship between ATPase and Al tolerance has not been studied in bacteria. Thus far, a relationship between ATPase and Al tolerance has only been seen in plants and yeast, e.g. the ALR1 (aluminium resistance) gene encodes the major Mg^{2+} uptake system in yeast (Lee and Gardner, 2006), also the potential role of vacuolar H^{+}-ATPase and the F-ATPase in Al and NaCl tolerance in wheat (*Triticum aestivum* L.) (Hamilton, 2002). Here we have characterized a new

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ATPase belonging to the PP-loop ATPase superfamily from *Anoxybacillus gonensis* G2 (Belduz *et al*., 2003) (for more information see: http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi).

Materials and Methods

Substrates, chemicals, and strains

ATP-2Na, lactic dehydrogenase, pyruvate kinase, and NADH were obtained from Sigma Chemical Co. (USA). Phosphoenol-pyruvate was purchased from Fluka (Germany). All protein chromatography matrices were purchased from Amersham Pharmacia (Germany). Wizard Genomic DNA Purification kit, dNTP and all restriction enzymes were purchased from Promega. Hot Gold Star DNA polymerase was obtained from Eurogentec (Belgium). QIAGEN Plasmid Isolation kit, PCR QIAquick Purification kit were obtained from QIAGEN (Germany). Protein Assay Kit and protein marker were obtained from Bio-Rad (UK). TOPO/TA Cloning kit (Invitrogen, USA), pET11a+ (Novagen, Germany) and pUC18 (MBI Fermentas, Lithuania) were used in cloning studies. The strain G2 was isolated from Gonen hot spring in the province of Balikesir in Turkey and identified as *Anoxybacillus gonensis* G2 NCIMB 13933 (Belduz *et al*., 2003). *Escherichia coli* BL21(DE3) (Novagen) and TOP10 (Invitrogen) strains were used in this study.

Cloning the *G2alt* gene

We constructed a genome library for *Anoxybacillus gonensis* G2 using the pUC18 plasmid and obtained a construct carrying the *alt* gene. The coding region of the *G2alt* gene was amplified by the polymerase chain reaction (PCR) with Hot Gold Star DNA polymerase (Eurogentec) using the forward primer 5′-CAT*ATg*AAAAAggAAAAAgCAgTTG-3′, (*Nde*I site is underlined) and the reverse primer 5′-ggAgCAAATgTgCgA*TgA*ggATCC-3′ (*Bam*HI site is underlined). The PCR product was purified with the PCR QIAquick Purification kit (QIAGEN) and subcloned into the TOPO-T/A cloning vector (Invitrogen) generating pTOPO-*G2alt*. The pTOPO-*G2alt* was cut by *Bam*HI/*Nde*I and was cloned into the expression vector pET11a(+) cut by the same restriction enzyme (pET11a+/*G2alt*). The sequence was verified by DNA sequencing of the final construct. *E. coli* TOP10 was used for subcloning procedures and *E. coli* BL21(DE3) was used for expression of the *alt* gene. *E. coli* (DH5α-MCR) was used to show Al tolerance.

Screening of Al tolerance in *E. coli* transformant

To determine maximum tolerance concentration (MTC) of Al, the pET11a+/*G2alt* vector was used to transform *E. coli* (DH5α-MCR), which was then cultured in LB liquid medium containing different concentrations of Al (from 10 μM to 5 mM), ampicillin (50 mg/ml), 0.5 mM IPTG and adjusted to pH 4.0 at 37°C. We used the untransformed *E. coli* (DH5α-MCR) grown under the same conditions as the control. MTC were calculated from the average of three independent experiments.

Overexpression and purification of G2ALT in *E. coli*

The *alt* gene was overexpressed using *E. coli* strain BL21(DE3) harboring pET11a+/*G2alt* grown at 37°C in LB medium containing 100 μg/ml carbenicillin. Cells at OD_{600}=0.6 were induced overnight with IPTG (1 mM final concentration). The cells were harvested by centrifugation and resuspended in 1X PBS buffer (1.4 M NaCl, 27 mM KCl, 101 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.3; 20 ml/L culture) and stored at -80°C. After repeated thawing and freezing cycles, the cells were sonicated (20 sec on and 20 sec off for 5 times, 30% amplitude, using Branson Sonicator, USA). The soluble protein fraction was separated from the insoluble and membrane proteins by centrifugation for 30 min at 14,000 rpm. Since the overproduced protein is expected to be thermostable, the soluble protein extract was incubated at 65°C for 30 min. The heat denaturated proteins were removed by centrifugation at 14,000 rpm for 30 min at 4°C. This was followed by cation and anion exchange chromatography on S-Sepharose Fast Flow (Amersham Pharmacia), 20 mM MES pH 6.0, and on Q-Sepharose Fast Flow (Amersham Pharmacia), 20 mM Tris pH 7.5 and pH 8.0, respectively. The selected column matrices were chosen as the predicted ioselectric point (pI) of the G2ALT protein is 6.94. After dialysing the protein extract against the corresponding buffer, 1 ml sample was loaded onto a 2.5×20 cm S-Sepharose column. The proteins were eluted using a stepwise gradient of NaCl (0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 M). The separation on the S-Sepharose gave the best results, and the G2ALT protein-containing fractions were pooled and concentrated using an Amicon filtration membrane (Ultra-15, cut off 10 kDa, Millipore, USA). This fraction was separated by size-exclusion chromatography on a Hiload Superdex200 16/60 column connected to an Akta Explorer system (Amershan Biosciences, Germany). The G2ALT protein-containing fractions were pooled and concentrated to 10 mg/ml. Protein concentration was determined using the BioRAD Protein Assay kit. Proteins were separated on 10% (w/v) SDS-polyacrylamide slab gels at room temperature. Coomassie Blue R-250 was used to visualize the protein bands on the gels. The final *A. gonensis* G2ALT fraction was judged to be pure, based upon the observation that there were no contaminating bands on a silver stained SDS-PAGE gel.

Determination of ATPase activity

Hydrolysis of ATP was monitored using a coupled spectrophotometric enzyme assay as described (Ogawa *et al*., 2000). The conversion of ATP to ADP and P_i is linked to the oxidation of NADH to NAD^+, and is monitored as a decrease in absorbance at 340 nm. Assays were carried out at 37°C in a spectrophotometer with a thermostated multicuvette holder (Secoman, France).

Purified G2ALT protein (2 μg) was incubated in 500 μl reaction buffer containing 50 mM HEPES, pH 7.4, 50 mM NaCl, 4 mM $MgCl_2$, 0.2 mM NADH, 0.5 mM phosphoenol-pyruvate, 18 units/ml lactic dehydrogenase and 24 units/ml pyruvate kinase. After preincubation of the assay mixture for 5 min at 37°C, the reaction was initiated by the addition of 0.5 or 1 mM ATP (final concentration). The decrease of optical density at 340 nm was continuously monitored.

Characterization of ATPase activity

The coupled spectrophotometric assay can not be used at higher temperatures because of the instability of lactic dehydrogenase and pyruvate kinase. For this reason the ATPase activity of the G2ALT protein was followed by measuring the released Pi from ATP by a method previously described (Goldenberg and Fernandez, 1966). The standard reaction buffer was 50 mM Trisl; pH 8.0, 400 mM NaCl, 4 mM $MgCl_2$, and 2 mM ATP.

The pH optimum for the ATPase activity was determined by changing the buffer within a pH range from 4.0 to 10.0. The following buffers were used: 50 mM sodium acetate buffer for pH 4.0-6.0, 50 mM HEPES for pH 7.0-8.0, 50 mM Tris for pH 8.0-9.0, and 50 mM glycine-NaOH buffer for pH 9.0-10.0. The final concentration of G2ALT was 10 μg/μl (stock concentration of the purified enzyme 1 mg/ml).

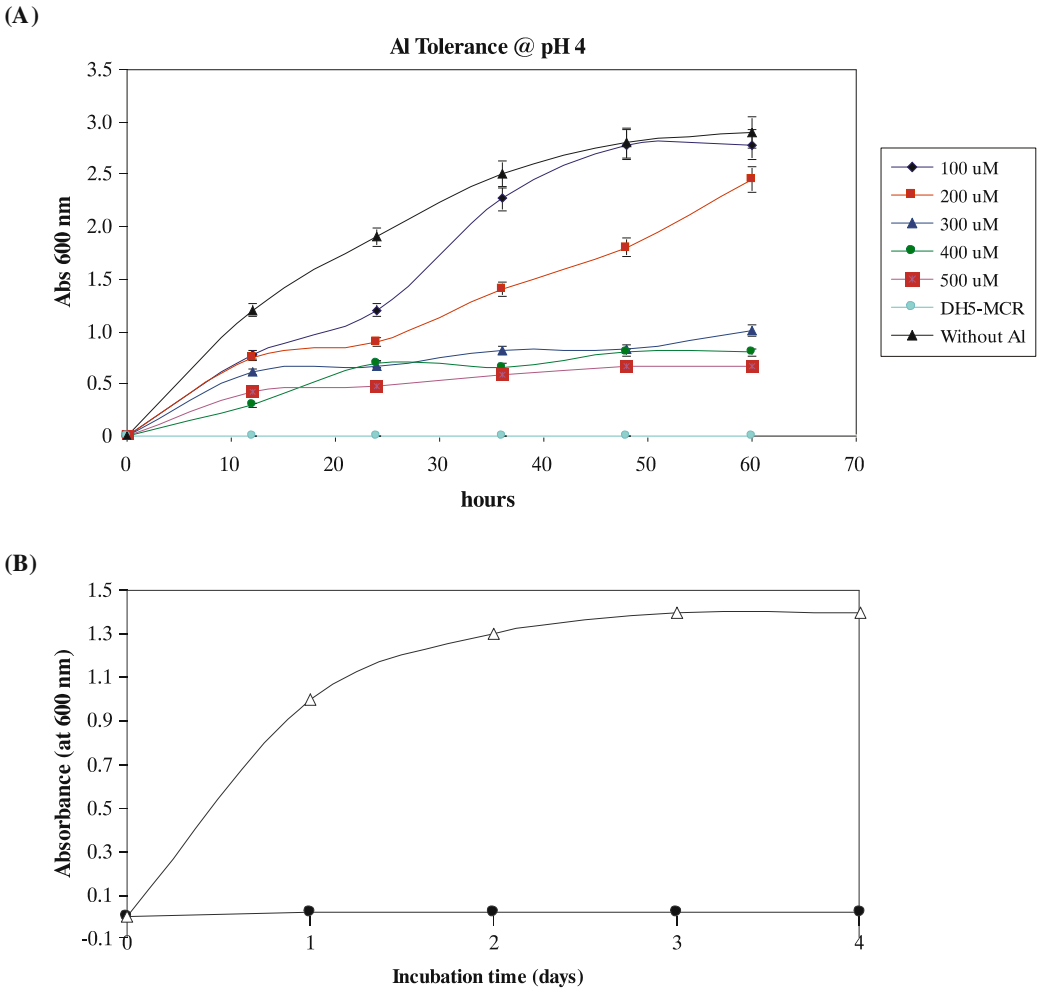

Fig. 1. (A) Growth patterns of *E. coli* (DH5α-MCR) and *E. coli* transformant with pET11a+/*alt* in medium containing 100, 200, 300, 400, and 500 μM of Al adjusted to pH 4.0 at 37°C, (B) Growth patterns of the transformed (-△-) and untransformed cells of (-○-) cultured in liquid medium containing 500 μM of Al adjusted to pH 4.5 at 37°C.

The activity was determined as the amount of released Pi after 15 min at 55°C, in a final volume of 500 μl. The optimum temperature for enzymatic activity was determined in the standard reaction buffer (pH 6.0) at 25, 35, 45, 55, 65, 75, 85, and 100°C. The results were expressed as percentages of the activity obtained at either the pH optimum or the temperature optimum in terms of mg released Pi/min/mg protein. Activities were calculated from the average of three independent experiments.

The kinetic parameters of the enzyme were determined at the pH and temperature optimum. Purified G2ALT was left to react in a 100 μl reaction mixture I (50 mM sodium acetate pH 6.0, containing 400 mM NaCl, 4 mM $MgCl_2$, 2 mM ATP). Aliquots of 20 μl were taken at different time points and the reaction was quenched by adding 500 μl of mixture II (10% w/v TCA, 1% w/v thiourea, 3% w/v Mohr's salt). The released Pi was quantified by addition of 50 μl of an ammonium molybdate reagent (4.2% ammonium molybdate in 4 N HCl). After 1 h incubation at room temperature, the inorganic phosphate released was calculated based on the absorbance standard curve established by KH_2PO_4 standards (Goldenberg and Fernandez, 1966).

The effect of three cations (Na^+, K^+, Mg^{2+}), the combinations of these cations (i.e. Na^+/K^+, Na^+/Mg^{2+}, and Mg^{2+}/K^+), other biologically relevant cations (Ag^+, Li^+, Cd^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, and Al^{3+}), cation chelating reagents (EDTA, sodium azide), reducing agent (β-mercaptoetanol), and of a common Na^+/K^+-ATPase inhibitor agent (ouabain) on the activity of the enzyme was determined as described above. The chloride salts were used for the metal ions, except for nitrate salt of Ag^+. The activity is expressed as a percentage of the activity determined for the enzyme in the absence of any activator/inhibitor. IC_{50} values for the different compounds were calculated.

Nucleotide sequence accession number

The sequence data for the *A. gonensis* G2 gene and protein have been assigned to GenBank accession no. ABY59726.

Results

Screening of Al tolerance in *E. coli* transformant

Both the untransformed and transformed *E. coli* cells could grow very well in the medium without Al at 37°C. Under the different concentrations of Al up to 500 μM, the recombinant

Fig. 2. Amino acid sequence alignment of *Geobacillus thermodenitrificans* NG80-2 (ABO66241), *Geobacillus kaustophilus* HTA426 (BAD75260), *Bacillus cereus* B4264 (ZP_02579119), *B. cereus* AH1134 (Q81G69), *B. cereus* G9842 (ZP_02586413), which are aluminium resistance proteins, *B. thuringiensis* str. Al Hakam (ABK84554), *B. cereus* AH187 (ZP_02253461), *B. thuringiensis* serovar *konkukian* str. 97-27 (YP_035565), which are possible aluminium resistance proteins, *Geobacillus kaustophilus* Queuosine biosynthesis protein QueC (Q5L1C0), *G.* sp. WCH70 exsB protein (EDT36055), and *A. gonensis* G2 aluminium tolerance protein (G2ALT). A perfectly conserved position and a well-conserved position are indicated by an asterisk (*) and a dot (•). A dash (-) represents the gap inserted to optimize the alignment, as well as the consensus fingerprint sequence for the PP-loop ATPase (grey box) .

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ABK84554        CETDFSGYPDCRDVFVKSLNVTLNLSMDYPFVIHTPLMWIDKAETWKLSDELGAFEFVRE 184
YP_035565       CETDFSGYPDCRDVFVKSLNVTLNLSMDYPFVIHTPLMWIDKAETWKLSDELGAFEFVRE 181
ZP_02253461     CETDFSGYPDCRDVFVKSLNVTLNLSMDYPFVIHTPLMWIDKAETWKLSDELGAFEFVRE 181
Q81G69          CETDFSGYPDCRDVFVKSLNVTLNLSMDYPFVIHTPLMWIDKAETWKLSDELGAFEFVRE 181
ZP_02586413     CETDFSGYPDCRDVFVKSLNVTLNLSMDYPFVIHTPLMWIDKAETWKLSDELGAFEFVRE 181
ZP_02579119     CETDFSGYPDCRDVFVKSLNVTLNLSMDYPFVIHTPLMWINKAETWKLSDELGAFEFVRE 181
G2ALT           CETDFSGYPDCRDVFIKSLNVTLNLAMDYQFVIHTPLMWLNKAETWKLADELGALEFVRN 181
EDT36055        CETDFSGYPDCRDIFIKSLNVTLNLAMDYQFVIHTPLMWLTKAKTWKLADELGAFDFVRT 240
BAD75260        CETDFSGYPDCRDVFIKSLNVTLNLAMDYEFVIHTPLMWLTKAETWKLADELGALEFIRT 184
Q5L1C0          CETDFSGYPDCRDVFIKSLNVTLNLAMDYEFVIHTPLMWLTKAETWKLADELGALEFIRT 181
ABO66241        CETDFSGYPDCRDIFIKSLNVTLNLAMDYPFVIHTPLMWLTKAETWKLADELGALEFVRT 183
                ************:*:*********:*** *********: **:****:*****::*:*

ABK84554        KTLTCYNGIIGDGCGECPACQLRKAGLDTYLQEREGASN- 223
YP_035565       KTLTCYNGIIGDGCGECPACQLRKAGLDTYLQEREGASN- 220
ZP_02253461     KTLTCYNGIIGDGCGECPACQLRKAGLDTYLQEREGASN- 220
Q81G69          KTLTCYNGIIGDGCGECPACQLRKAGLDTYLQEREGANN- 220
ZP_02586413     KTLTCYNGIIGDGCGECPACQLRKAGLDTYLQEREGANN- 220
ZP_02579119     KTLTCYNGIIGDGCGECPACQLRKAGLDTYLQEREGANN- 220
G2ALT           KTLTCYNGIIADGCGECPACVLRKRGLDQYMNEKKGANVR 221
EDT36055        KTLTCYNGIIADGCGECPACVLRRRGLEEYMKEKEGANQL 280
BAD75260        KTLTCYNGIIADGCGECPACALTKRGLDEYLREKAEVESR 224
Q5L1C0          KTLTCYNGIIADGCGECPACALTKRGLDEYLREKAEVESR 221
ABO66241        KTLTCYNGVIADGCGECPACVLRKRGLEEYLQEKAGVKAR 223
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Fig. 2. Continued

cells showed good growth whereas the untransformed cells showed no growth (Fig. 1A). Figure 1B shows the growth of transformed and untransformed cells cultured in liquid media containing 500 μM Al at pH 4.5. These results suggest that the transformant acquired Al tolerance by carrying the DNA fragment that confers Al tolerance to *A. gonensis* G2. That is, the recombinant plasmid, pET11a+/*alt*, has the DNA fragment encoding a protein that endows *E. coli* with Al tolerance.

Similarity analysis of the protein

G2ALT is highly similar to other proteins responsible for aluminium resistance. G2ALT has 59% to 86% identity with twenty-four homologous proteins in the NCBI-BLAST search. According to protein BLAST similarity results, G2ALT is similar to *Geobacillus thermodenitrificans* NG80-2 aluminium resistance protein (86%), *G. kaustophilus* HTA426 aluminium resistance protein (86%), *Bacillus cereus* B4264 aluminium resistance protein (84%), *B. cereus* G9842 aluminium resistance protein (84%), *B. thuringiensis* str. Al Hakam (83%), *B. cereus* AH187 possible aluminium resistance protein (83%), and *B. thuringiensis* serovar *konkukian* str. 97-27 aluminium resistance protein (83%). G2ALT also has 87% similarity to ALU1-P, which is associated with aluminium tolerance in *A. viscosus* (Jo *et al*., 1997). The most similar proteins to G2ALT are

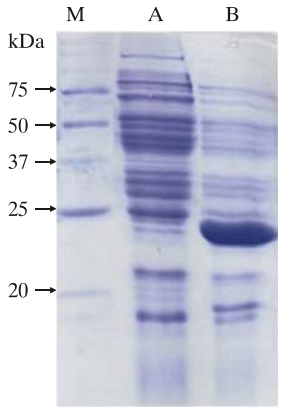

Fig. 3. SDS-PAGE analysis of *A. gonensis* G2ALT protein profile. A, total cell lysate from *E. coli* BL21(DE3) as control; B, *E. coli* BL21(DE3)/pET11a(+)/*alt* after IPTG induction; M, molecular marker.

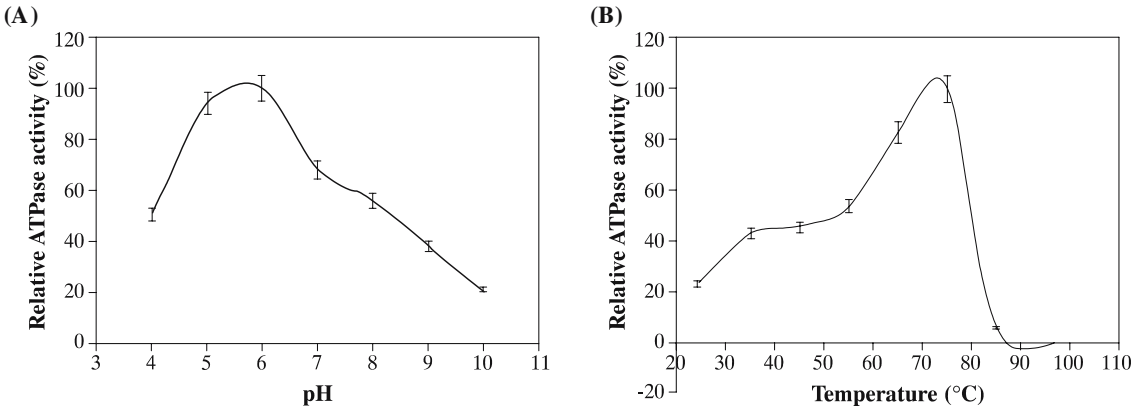

Fig. 4. pH and temperature effects on the ATPase activity of ALT (A and B, respectively). The enzyme activity was measured at the indicated temperatures and pHs in the presence of 10 mM Mg^{2+} and 400 mM NaCl. Each point represents the average of three experiments. Each symbol and its bar represent Mean±SD.

shown in Fig. 2.

Cloning, expression, and purification of G2ALT

The *G2alt* gene, 666 bp, was cloned into pET11a+. The expression profiles of the clone were checked at different time periods (t: 0, 2, 4, and overnight), and at different temperatures (28 and 37°C) in *E. coli* strains BL21(DE3) and C43 (DE3). The highest expression level was found for overnight incubation, which was subsequently used for overproduction. The overproduced enzyme is a soluble cytoplasmic protein. We used heat treatment to denature the proteins from *E. coli* and removed them by centrifugation. After a scouting of

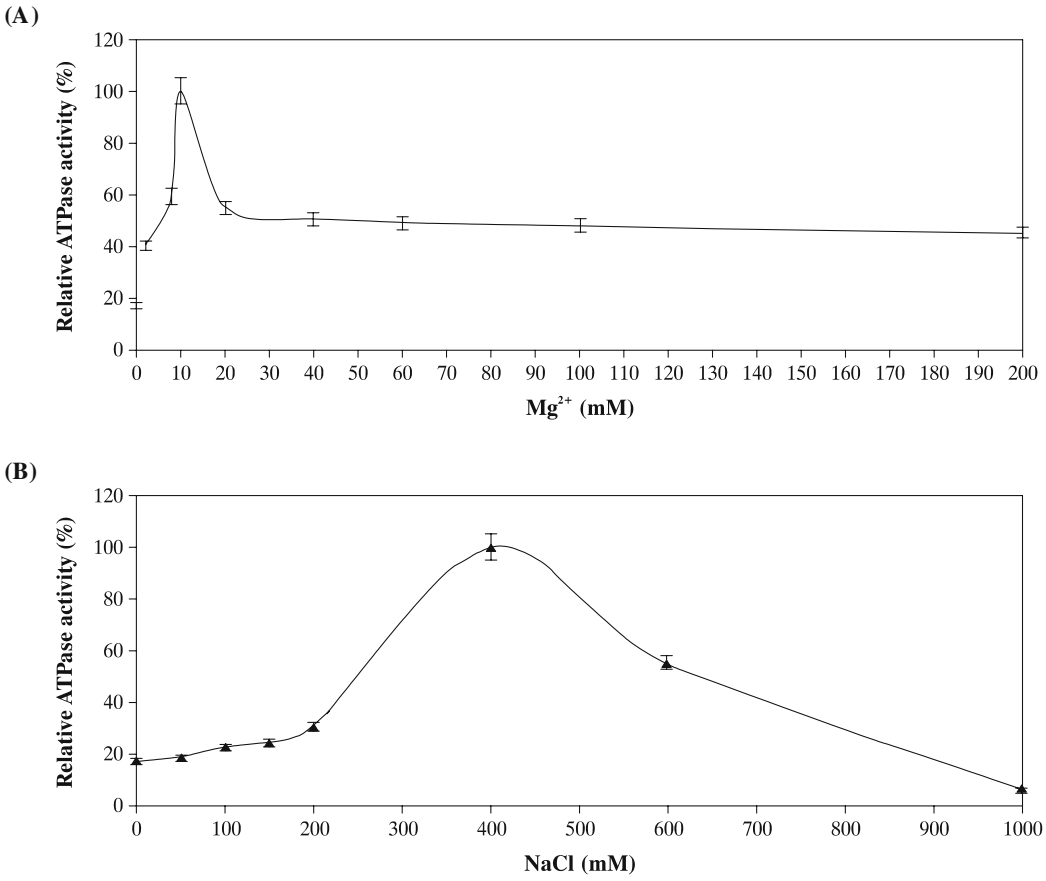

Fig. 5. Effect of magnesium and salt on ATPase activity (A and B, respectively). Each symbol and its bar represent Mean±SD.

different ion exchange chromatography techniques and conditions, we concluded that separation of the protein extract on a S-Sepharose column at pH 6.0 gave the best results. As a final purification step, the concentrated G2ALT-containing fractions were subjected to a Hiload Superdex 16/60 column. The protein was eluted at a volume which corresponds to 150 kDa, which reveals a hexamer in solution. The final yield was 3 mg of G2ALT per 2 L of culture. The purified protein

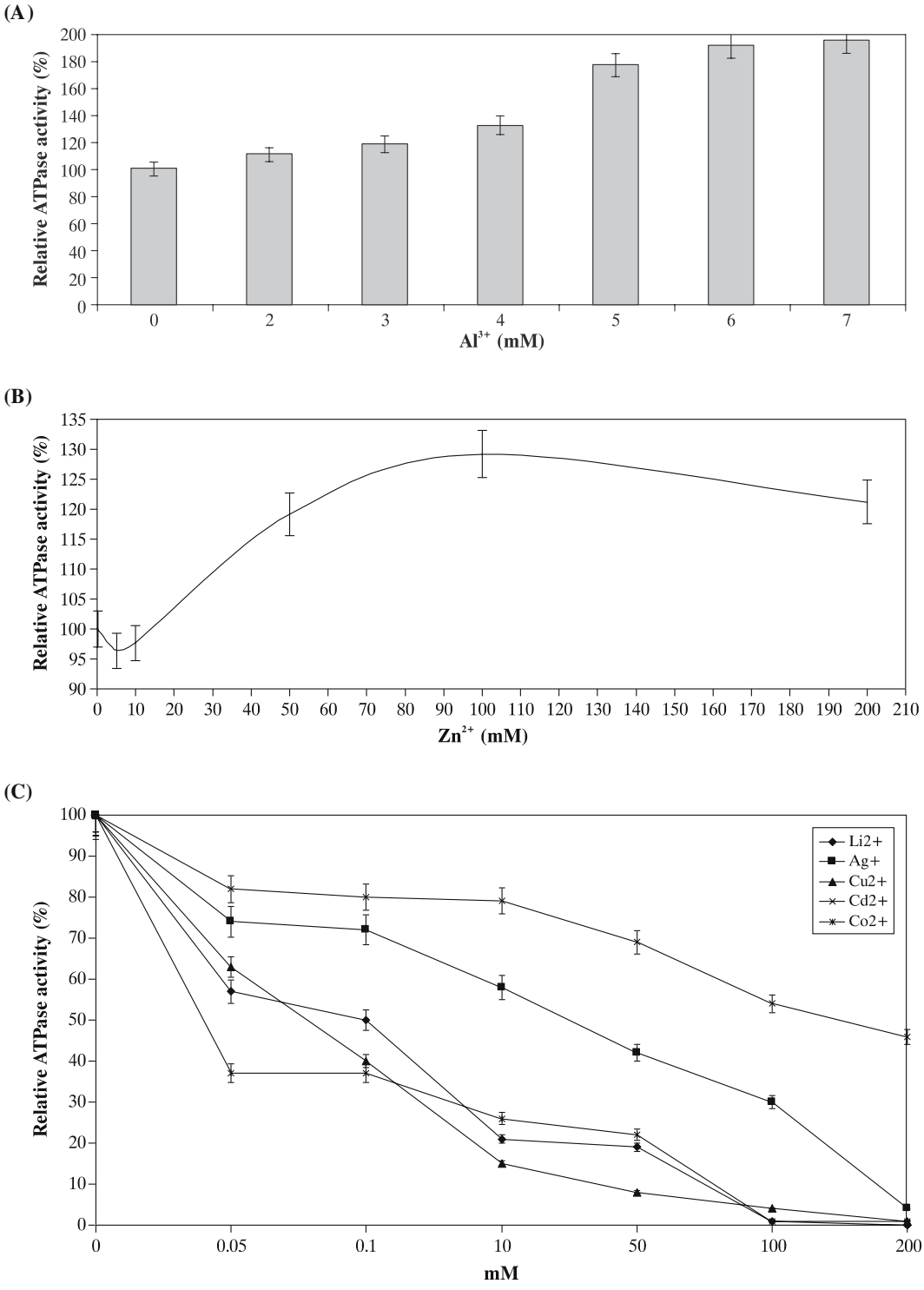

Fig. 6. Effects of mono-, di-, and trivalent cations on the enzyme activity. ATPase activity was measured in the presence of different concentrations of the cations. (A) Effect of different aluminium concentrations on the enzyme activity, (B) Effect of zinc ions, and (C) of other cations on the enzyme activity. The initial activity was defined as 100%. Each symbol and its bar represent Mean±SD.

migrated as a single band of 24.5 kDa *M*r on an SDS-PAGE gel, in agreement with the predicted molecular mass (Fig. 3). The N-terminal sequence of the overproduced and purified enzyme was MKKEKAVVVF as determined by Edman degradation and this corresponds with the translated DNA sequence.

Kinetic characterization of G2ALT

The highly conserved nucleoside triphosphate binding motif SGGQDST, found in the PP-loop superfamily ATPases, is also conserved in the G2ALT protein (Fig. 2). Based on this observation, the ATP hydrolysing properties of G2ALT were tested. The specific ATPase activity of purified G2ALT was measured in a standard coupled enzyme assay under a variety of conditions according to (Ogawa *et al*., 2000). As a preliminary characterization, its ATPase activity was measured at 37°C and found to be 0.14 μM Pi/min/mg of protein. Because of the thermophilic properties of *A. gonensis* G2ALT, a temperature profile of the ATPase activity was determined. Unfortunately, the enzymes of the coupled enzyme assay denature and precipitate at higher temperatures and therefore a colorimetric assay was substituted to measure the released Pi from ATP hydrolysis. The optimum pH is 6.0 (Fig. 4A) and the apparent optimal temperature is 73°C (Fig. 4B). The kinetic parameters of the G2ALT protein were determined at the pH and the apparent optimal temperature of 6.0 and 73°C, respectively. The K_m is 10±0.55 μM and the value of V_{max} is 26.81±0.13 mg Pi released/min/mg enzyme. The K_m and V_{max} data were calculated by Lineweaver-Burk analysis.

Effects of cations on the ATPase activity

ATPase activity was measured in the presence of various mono-, di-, and tri-valent cations at various concentrations. The optimum concentration of Mg^{2+} was determined by titration experiments to be 10 mM (Fig. 5A). Higher Mg-ATP concentrations did not have a stimulatory effect. Na^+ concentration also strongly influenced the ATPase activity, with an optimum at 400 mM (Fig. 5B), but higher concentrations reduced the activity. K^+ had a similar effect on the activity as Na^+ for which 50 mM showed the greatest effect (data not shown). However, the influence of the combination of Na^+ and Mg^{2+} was more than expected because the activity was higher than when using only one cation. This probably means that the two ions have a different but cumulative effect on the activity. Zn^{2+} and Al^{3+} moderately supported the ATPase activity; other biologically relevant cations including Ag^+, Co^{2+}, Cd^{2+}, Cu^{2+}, and Li^{2+} did not. The addition of 7 mM Al^{3+} doubled the activity and the addition 100 mM Zn^{2+} surprisingly increased it 1.2 fold (Figs. 6A and B, respectively). Addition of 5 μM Co^{2+} and 1 mM Li^{2+} reduced the activity by half and concentrations of 100 mM and higher had an inhibitory effect (Fig. 6C). The activity was reduced to 40% in the presence of 50 μM Cu^{2+}, while 200 mM Cu^{2+} completely inhibited the enzyme. Higher concentration of Mn^+ reduced the activity by 70% (data not shown). Cadmium had little effect, as concentrations up to 10 mM only reduced the activity by 20% (Fig. 6C). At concentrations higher than 50 mM, the activity was gradually decreased but not completely inhibited. Similarly, Ag^+ reduced the activity gradually, with the activity reduced by half at 30 mM. The effects of other commonly used inhibitors of enzyme activity were also tested (Fig. 7). G2ALT was completely inhibited by β-mercaptoethanol and ouabain, with an IC_{50} of 5 mM and 94 μM, respectively. The activity was reduced by 25% at a concentration of 100 μM EDTA, but its IC_{50} value was 68 mM. Sodium azide gradually reduced the activity. Although its IC_{50} value was as low as 80 μM, enzyme activity was not completely abolished (residual acitivity of 14%) at the higher concentrations of 50-200 mM.

Discussion

The characterization of the ATPase activity of G2ALT from a thermophilic bacterium, *A. gonensis* G2, and the effects of environmental conditions on its biological functioning are reported here, for the first time. It is known that aluminium ions are highly soluble in acidic environments (Fischer *et al*., 2002); however, *A. gonensis* G2 (AgG2) doesn't grow at acidic pH. Therefore, the aluminium resistance of AgG2 could not be shown directly. In *Arthrobacter viscosus*, the homologous gene is designated *alu1-p* and is associated with an aluminium tolerance phenotype. G2ALT is similar to *alu1-p* with 87% similarity. *A. gonensis* G2 is different from *A. viscosus* in having its optimum growth pH from 6 to 10 (Belduz *et al*., 2003). These pH averages are not suitable for analysing aluminium resistance in bacteria because aluminium is toxic at a pH lower than 5. To determine the MTC of Al, we used the transformed and untransformed *E. coli (DH5α-MCR)* in an LB liquid medium containing different concentrations of Al. Up to 500 μM of Al, the transformed cells showed good growth. This value shows that the *alt* gene of *A. gonensis* G2 is responsible for Al tolerance and conditions a higher tolerance than *A. viscosus* ALRJJ6, which has its MTC at 200 ppm under the same conditions (Jo *et al*., 1997). The *alu1-p* gene that is responsible for aluminium tolerance of *A. viscosus*, has not been completely sequenced. The sequence of the incomplete ORF of *alu1-p* from *A. viscosus* and the *G2alt* gene from *A. gonensis* G2 are highly similar. Thus, the studies on the characterization of the *G2alt* gene have provided important information towards understanding Al tolerance.

The amino acid sequence of G2ALT has a high similarity with proteins that are responsible for aluminium resistance. Moreover, according to protein BLAST similarity results, this gene is similar to proteins belonging to the PP-loop ATPases superfamily composed of *B. cereus* ATCC 14579, *B. cereus* E33L, and *B. thuringiensis* serovar *konkukian* str. 97-27 (91%), *G. kaustophilus* HTA426 (90%), *A. viscosus* alu1-p (87%), *C. acetobutylicum* ATCC 824 (85%), and *S. mutans* UA159 (83%). Some of these proteins were annotated by TIGR as proteins responsible for aluminium resistance (for more information see: http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi). The deduced amino acid sequence of the *G2alt* gene was aligned with some of these protein sequences using CLUSTAL W (http://www.ebi.ac.uk/clustalw). The fingerprint sequence, ^{11}SGGXDST^{17} (where X is any hydrophobic amino acid), a feature of the ATP-utilizing enzymes belonging to the PP-loop ATPase superfamily (Fig. 2) was found to be conserved.

G2ALT was overproduced as a soluble polypeptide of 221 amino acids in *E. coli* and characterized as an ATPase. The most widely used coupled enzyme assay could not be used to study the ATPase activity because the protein originated from a thermophilic bacterium. Therefore, a colorimetric assay

based on molybdate precipitation to measure the released Pi from ATP hydrolysis was used (Goldenberg and Fernandez, 1966). The apparent optimal temperature for the ATPase activity of the enzyme is 73°C, which is much higher than the optimum growth temperature of 55°C of the bacterium. This result is comparable to the glucose isomerase (GI) of the same bacterium, which has a temperature optimum of 85°C (Karaoglu, 2010). This phenomenon was also found for other ATP hydrolysing enzymes, irrespective of whether they originated from thermophilic or mesophilic bacteria. The maximum ATPase activity of GroEL, the *E. coli* chaperone, was determined to be 49°C (Melkani *et al*., 2003), of the p97-VCP AAA-type ATPase from *E. coli* to be 50°C (Song *et al*., 2003), of the UvrB from *Thermus thermophilus* HB8 to be 65°C (Kato *et al*., 1996), of the CopA, P-type ATPase from extremophile *Archaeoglobus fulgidus* to be 75°C (Stoyanov *et al*., 2003), of a soluble P-type ATPase (MJ0968) from the Archaeon *Methanococcus janashii* to be 50°C (Ogawa *et al*., 2000) and of another P-type ATPase (MJ1226p) from the same archaeon to be 95°C (Morsomme *et al*., 2002).

The optimum pH for the ATPase from *A. gonensis* G2 is 6.0, which is similar to other P-type ATPases and F-ATPase from bacteria and yeast (Song *et al*., 2003; Magalhaes *et al*., 2005). The protein exhibits Michealis-Menten kinetic properties of ATP hydrolysis at 73°C. The ATPase activity was dependent on the presence of magnesium and sodium, like other mammalian and bacterial P-type ATPases (Magalhaes *et al*., 2005). The purified G2ALT exhibited an ATPase activity with K_m and V_{max} values of 10±0.55 μM and 26.81±0.13 mg Pi/min/mg enzyme respectively, at an optimal pH of 6.0, and in the presence of 10 mM Mg^{2+} and 400 mM Na^+ ions. As comparisons with other enzymes that have an ATP-binding P-loop domain, *A. aeolicus* TilS (lysidine synthetase) has a K_m of 19.4±2.1 μM and *M. tuberculosis* NAD^+ synthase has a K_m of 6.19 μM and V_{max} of 9.6 nmol/min/mg of protein. ATPase activity of ALT from *A. gonensis* G2 was similar to these enzymes (Cantoni *et al*., 1998; Nakanishi *et al*., 2005).

It is known that metal ions have an important role in maintaining enzyme activity and stabilizating the structure (DiTusa *et al*., 2001; Colak *et al*., 2005). The influence of some metal ions on the *A. gonensis* G2 ATPase activity was examined by using various metal chloride salts at various concentrations. Purified G2ALT was activated by Zn^{2+} and Al^{3+}. However, Al^{3+} inhibited the H^+-ATPase activity of both the plasma membrane (AHA3) and the lysosomal proton pump, even at the very low concentrations of 10 to 50 μM (Zatta *et al*., 2000; DiTusa *et al*., 2001). On the other hand, it was reported that Al^{3+}, at a 50 μM concentration, increases the Na^+/K^+-ATPase activity in the rat brain by about 60% compared to the controls (Zatta *et al*., 1995). The activation of the ALT protein with Al^{3+} is unique. At higher concentrations, Li^+, Cu^{2+}, and Co^{2+} completely inhibited the activity of the enzyme. The reduction of the ATPase activity in the presence of EDTA can be attributed to its metal chelating effect (Fig. 7) (Colak *et al*., 2005). Sodium azide and ouabain, common inhibitors of most ATPases and sodium pumps, also inhibit the G2ALT enzyme (Schneider *et al*., 1998). The effect of ouabain inhibition also supports that the ATPase activity of G2ALT needed Na^+ ions. β-Mercaptoethanol completely inhibited the activity of enzyme due to the reduction of the disulfide bridges.

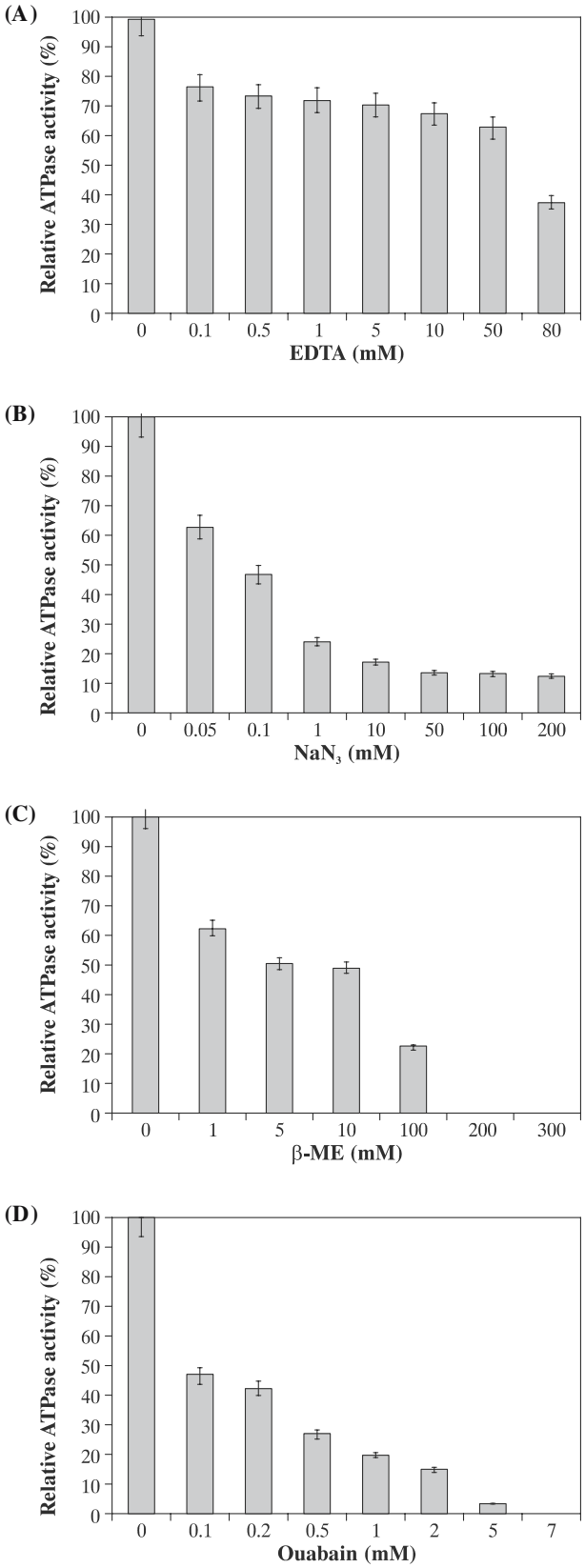

Fig. 7. Inhibitory effect of (A) EDTA, (B) NaN_3, (C) β-mercaptoethanol, and (D) ouabain on the ATPase activity. The initial activity was defined as 100%. Each symbol and its bar represent Mean±SD.

The sequence similarity analysis for G2ALT from AgG2 also supported that the G2ALT protein is responsible for aluminium tolerance. In *Arthrobacter viscosus*, the homologous gene is designated *alu1-p* and is associated with an aluminium tolerance phenotype. G2ALT is similar to alu1-p with 87% similarity. Therefore, based upon similarity searches and MTC experiments, this protein is responsible for aluminium tolerance in *A. gonensis* G2. The enzymatic properties of G2ALT supported this structural information.

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