The long Q-loop of Escherichia coli cytochrome bd oxidase is required for assembly and structural integrity

Alexander Theßeling, Sabrina Burschel, Daniel Wohlwend and Thorsten Friedrich

Institut für Biochemie, Albert-Ludwigs-Universität Freiburg, Germany

Correspondence
T. Friedrich, Albert-Ludwigs-Universität, Institut für Biochemie, Albertstr. 21, D-79104 Freiburg, Germany
Tel: +49 (0)761 203 6060
E-mail: Friedrich@bio.chemie.uni-freiburg.de

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Cytochrome bd-I oxidase is a terminal reductase of bacterial respiratory chains produced under low oxygen concentrations, oxidative stress, and during pathogenicity. While the bulk of the protein forms transmembrane helices, a periplasmic domain, the Q-loop, is expected to be involved in binding and oxidation of (ubi)quinol. According to the length of the Q-loop, bd oxidases are classified into the S (short)- and the L (long)-subfamilies. Here, we show that either shortening the Q-loop of the Escherichia coli oxidase from the L-subfamily or replacing it by one from the S-subfamily leads to the production of labile and inactive variants, indicating a role for the extended Q-loop in the stability of the enzyme.

Keywords: assembly; bd-I oxidase; Escherichia coli; Q-loop; quinol binding

Cytochrome bd-I oxidases are terminal oxidases of many prokaryotes, including several pathogens [1–3]. The enzyme complex is not related to the well-characterized family of heme-copper oxidases [4,5]. The bd oxidases couple (ubi)quinol oxidation and release of protons to the periplasmic side with proton uptake from the cytoplasmic side to reduce dioxygen to water. In doing so, bd oxidases contribute to the generation of the protonmotive force by vectorial charge transfer [6,7]. Universally, bd-I oxidase is made up of two major subunits CydA and CydB that share the same fold comprising two four-helix bundles and an additional cytoplasmic helix (Fig. 1). CydA harbors the cofactors, namely heme b558, b595, and d. Heme b558 accepts electrons from the quinol and transfers them to the heme active site composed of heme b595 and d where dioxygen is reduced to water [1,8]. In addition, CydA contains a globular domain located on the periplasmic side between transmembranous (TM) helices 6 and 7 (Fig. 1) [9]. This region, termed Q-loop, is expected to be involved in binding and oxidation of the substrate (ubi)quinol (see below) [9–11].

It was found that bd oxidases contain a third subunit, called CydX in Escherichia coli and CydS in Geobacillus thermodenitrificans [12–14]. CydX consists of 30–40 amino acids, and it is encoded by the cyd operon. In the oxidase, it is located at the interface of TM helices 1 and 6 of CydA probably stabilizing the active heme center (Fig. 1). Surprisingly, E. coli bd oxidase contains a fourth subunit called either CydY or CydH that is not encoded in the cyd operon but derives from the orphan gene ynhF [15,16]. CydY is located in a hydrophobic cleft shaped by TM helices 1 and 9 of CydA and is absent in the G. thermodenitrificans enzyme. Because CydY blocks the dioxygen entry site present in both enzymes, the E. coli enzyme contains an additional unique substrate channel leading from CydB to heme d (Fig. 1). In accordance with the different positions of the dioxygen binding sites, the positions of heme b595 and d are interchanged in the E. coli enzyme with respect to the G. thermodenitrificans oxidase [15,16].

The Q-loop of CydA is in close proximity to heme b558, the primary electron acceptor, implying a function

Abbreviations
TM, transmembranous; HDX-MS, Hydrogen/deuterium exchange mass spectrometry.
in quinol oxidation [14–16]. Due to the presence of a short (S) or a long (L) Q-loop, the family of \textit{bd} oxidases is classified into the S- and L-subfamilies [1]. The Q-loop of the \textit{bd} oxidases from the S-subfamily (e.g., \textit{G. thermodenitrificans}) has a length of about 70 amino acid residues, while the members of the L-subfamily (e.g., \textit{E. coli}) contain a C-terminal extension of about 60 amino acid residues (Fig. 1) [1,14–17]. The N-terminal region of the Q-loop shows a higher amount of conserved amino acid residues than the C-terminal portion [10,11]. Recent structural data imply that the N-terminal domain of the Q-loop is intrinsically flexible and that binding of the quinone-site inhibitor aurachin D reduces its flexibility, yet still without leading to a defined structural conformation. In contrast, the additional C-terminal extension of the Q-loop present in the members of the L-subfamily is rather rigid and extends all over the periplasmic surface of CydA to the CydAB interface [15,16]. This implies a functional role for the N-terminal domain of the Q-loop and a structural role for its C-terminal extension that is only found in members of the L-subfamily.

Here, we truncated the Q-loop of the \textit{E. coli} \textit{bd}-I oxidase as well as changed the enzyme into a \textit{bd} oxidase of the S-subfamily. Truncations were made based on structure comparison between the \textit{E. coli} and the \textit{G. thermodenitrificans} enzyme (Fig. 1). It turned out that all mutations led to the production of variants without the active heme center and consequently to an inactive and unstable oxidase that could not be purified by chromatographic means. Thus, the C-terminal extension of the Q-loop indeed seems to stabilize the entire enzyme complex emphasizing its structural role.

Materials and methods

Construction of expression plasmids

Three expression plasmids were generated from plasmid pET28b(+) \textit{cydA} by that encodes the \textit{E. coli} \textit{bd}-I oxidase [12]. The newly designed plasmids were designated as \textit{Q}_{\text{short}}, \textit{Q}_{\text{helix}}, and \textit{Q}_{\text{Geo}}. The plasmid \textit{Q}_{\text{short}} encodes an oxidase that lacks amino acid residues 250 to 384, thus, the entire Q-loop. To link TM helices 6 and 7 of CydA, nucleotides encoding a linker sequence ‘EDERP’ that connects these two helices in the \textit{G. thermodenitrificans} \textit{bd} oxidase were inserted. The plasmid \textit{Q}_{\text{helix}} lacks the nucleotides encoding amino acid residues 258–384. Here, only the
N-terminal helix of the *E. coli* Q-loop is encoded in the plasmid and nucleotides coding the linker sequence ‘DGDGDGP’ were inserted that were predicted to form a flexible loop preserving the rest of the structure [18]. The intention behind this approach was that the N-terminal helix of the *E. coli* Q-loop might be needed to stabilize CydA. The plasmid *Q*geo encodes the *E. coli* oxidase equipped with the Q-loop of the *G. thermodenitrificans* oxidase. The *E. coli* nucleotides encoding amino acid residues 258–384 were deleted, and nucleotides encoding the *G. thermodenitrificans* oxidase from position 258–317 were inserted. The resulting sequences are shown in Table S1. Oligonucleotides *Q*short_fwd and *Q*short_rev were used to generate plasmid *Q*short, and oligonucleotides *Q*helix_fwd and *Q*helix_rev were used to generate *Q*helix. The forward and reverse oligonucleotides share homologous regions to simplify recombination of the linear fragment. To generate a chimera of the *E. coli* bd-I oxidase comprising the Q-loop of the *G. thermodenitrificans* enzyme, the sequence coding the *G. thermodenitrificans* Q-loop was amplified from the synthetic plasmid pEX-A128 (Table S2; Eurofins Genomics, Ebersberg, Germany) containing the corresponding sequence with the oligonucleotides *Q*geo-insert_fwd and *Q*geo-insert_rev. The resulting linear fragment was used as primer in a second PCR to amplify the pET28b(+)*cydAhisBX Q*helix plasmid. Hundred and fifty nanogram oligonucleotide and 70 ng pET28b(+)*cydAhisBX Q*helix were used. The sequences of the oligonucleotides (all from Sigma-Aldrich, München, Germany) are listed in Table S3. All PCR products were digested with *Dpn*I and purified by agarose gel electrophoresis. The KOD polymerase (Merck Millipore, Darmstadt, Germany) was used according to manufacturer’s specifications. Newly generated vectors were checked by sequencing (GATC Biotech, Konstanz, Germany). Restriction enzymes were obtained from Thermo Fisher Scientific (Darmstadt, Germany) or Merck Millipore. All plasmids used in this work are listed in Table S4.

**Expression strains and cell growth**

*E. coli* strain CBO was used for electroporation [12]. This strain is a derivative of *E. coli* strain C43(DE3) chromosomally lacking *cydABX* and *appBCX* (designated: C43(DE3)recA, *cydABX, appBCX*). Thus, CBO lacks the genes of both *bd*-type oxidases leaving cytochrome *bo3* as the only respiratory oxidoreductase. This strain was individually transformed with pET28b(+), pET28b(+)*cydAhisBX* (called WT hereafter), pET28b(+)*cydAhisBX Q*short* (called *Q*short hereafter), pET28b(+)*cydAhisBX Q*helix* (called *Q*helix hereafter), or pET28b(+)*cydAhisBX Q*geo* (called *Q*geo hereafter) and grown aerobically in 2 L baffled flasks containing 800 mL LB medium at 37 °C. Gene expression was induced at an OD600 of ~2 by an addition of 0.4 mM IPTG. Cells were harvested in the late exponential phase and stored at −80 °C.

**Membrane preparation and detergent extract**

Frozen cells (15–20 g wet weight) were suspended in the fivefold volume 50 mM MOPS, 100 mM NaCl, 0.5 mM PMSF, pH 7.0 and disrupted by single pass through a French pressure cell (SLM Alminco) at 17 000 psi. Cell debris was removed by centrifugation at 9500 g for 20 min. Cytoplasmic membranes were sedimented by ultracentrifugation of the cleared lysate at 250 000 g for 75 min and subsequently suspended in 20 mM MOPS, 20 mM NaCl, 0.5 mM PMSF, pH 7.0 to a final protein concentration of 10 mg·mL⁻¹. Membrane proteins were solubilized by incubating the membrane suspension 90 min with 1% LMNG (Anatrace, Maumee, OH, USA) under mild stirring. Non-solubilized material was sedimented by ultracentrifugation at 250 000 g for 15 min.

**Redox difference spectra**

UV/Vis absorption spectra were recorded using a Tidas II Diode Array Spectrometer (J&M Analytik AG, Essingen, Germany). First, a spectrum of the air-oxidized membranes was recorded. The same sample was then reduced by an addition of a few grains of dithionite, and the spectrum of the reduced sample was recorded using the same set of parameters. The spectrum of the oxidized sample was subtracted from that of the reduced sample resulting in the dithionite-reduced minus air-oxidized difference spectra. The air-oxidized and the ferricyanide-oxidized spectra exhibited no difference.

**Oxidase activity**

The activity of the oxidases in membranes from the parental strain and the mutants was determined by measuring the NADH:oxygen oxidoreductase activity with an oxygen electrode (oxygraph+, Hansatech) at 30 °C. Five microliter membrane suspensions (~50 mg·mL⁻¹) were added to 2 mL buffer (20 mM MOPS pH 7.0, 20 mM NaCl). Strain CBO is lacking both *bd*-type oxidases. When indicated, the activity of the *bo3* oxidase was inhibited by an addition of 1 mM KCN to the buffer. The reaction was started by an addition of 5 μL NADH (0.5 mM). The rates were corrected for the nonenzymatic rate that was <1% of the rate of the enzymatic reaction. Each data point was assayed in triplicates from three different biological samples.

**Western blot analysis**

SDS/PAGE was performed according to von Jagow and Schägger [19]. Subsequently, proteins were electroblotted onto 0.45 μm pore size PVDF membrane (Schleicher and Schüll, München, Germany) according to Ref. [20]. The mouse anti-His-4 antibody was purchased from Merck Millipore and the secondary goat anti-mouse IgG, AP conjugated, from Qiagen (Hilden, Germany).
Attempts to purify bd oxidase variants

Cytoplasmic membranes were prepared as described above. All steps were carried out at 4°C. Membrane proteins were solubilized by incubating the membrane suspension 1 h with 1% LMNG (Anatrace). Nonsolubilized material was sedimented by ultracentrifugation at 250 000 g for 15 min. The supernatant was applied to a 1 mL HisTrap HP (GE Healthcare, Freiburg, Germany) column equilibrated in 50 mM MOPS, 100 mM NaCl, 0.03 mM PMSF, 0.003% LMNG, pH 7.0 (buffer A) containing in addition 20 mM imidazole, with a flow rate of 1 mL min⁻¹. The column was washed with buffer A containing 68 mM imidazole until the absorption dropped to the original baseline level. Bound proteins were eluted in a 20 mL linear gradient from 68 to 212 mM imidazole in buffer A.

Results and Discussion

Cells were grown under aerobic conditions in LB medium, and expression of the oxidase genes was induced by an addition of IPTG. Strain CBO/pET28b(+), grew to an OD of about 4.0 resulting in 6–9 g cells (wet weight) per L medium (Fig. 2). The strains with an insert in pET28b(+) grew significantly slower to an OD between 1.2 and 2.2 and yielded ~ 3–5 g cells (wet weight) per L medium (Fig. 2). This indicates that gene expression resulted in protein production. Cytoplasmic membranes from the various strains differed in color. Membranes from strain CBO/pET28b(+) cydA_hisBX had a brownish-reddish color, while those from the strains with pET28b(+) cydA_hisBX Q_short and pET28b(+) cydA_hisBX Q_heelix showed a light reddish color. Membranes from strains CBO/pET28b(+) cydA_hisBX Q_Geo and CBO/pET28b(+) were greenish-brown suggesting a higher amount of bo3 oxidase in the membranes.

The heme content of the cytoplasmic membranes was determined by UV/Vis spectroscopy (Fig. 3). The reduced-minus-oxidized difference spectrum of membranes from strain CBO/pET28b(+)cydA_hisBX (WT strain) showed the signals of heme bo558 at 430 nm and of heme bo595 at about 440 nm in the Soret region. The signal of heme d is superimposed by that of heme bo558 at 430 nm. Additional absorbance of heme bo558 was detected at 531 and 562 nm, of heme bo595/d at 594 nm and of heme d at 629 and at 655 nm (Fig. 3). Membranes from the strain with the empty plasmid and the strains with the plasmids coding for the bd variants just showed the signals of heme bo558, but lacked absorbance of heme bo595 and heme d (Fig. 3). The signals at 430, 531, and 562 nm contained contributions from the b-type hemes of bo3 oxidase [8] and succinate dehydrogenase [22].

The activity of the bd oxidase variants in the membrane was measured as NADH oxidase activity in the presence and absence of 1 mM KCN to inhibit bo3 oxidase [1,7] (Table 1). Strain CBO with the empty plasmid features the bo3 oxidase as sole respiratory oxidase with an IC₅₀ of 10 μM toward KCN [8]. Thus, the oxidase activity is nearly fully inhibited by an addition of 1 mM KCN. The bd oxidase has an IC₅₀ of 2 mM toward KCN [21] leading to an only minor inhibition under the applied conditions. Due to this, the activity of the strain producing the WT bd oxidase was only inhibited to 38% by KCN demonstrating the presence of a functional bd oxidase in the membrane. The oxidase activity of strains expressing Q_short, Q_heelix, and Q_Geo was also inhibited by 94% and 93% by KCN, respectively, indicating a complete lack of a functional bd oxidase. Noteworthy, the oxidase activity of Q_Geo in the absence of KCN is in the same range as the one obtained with the strain producing the WT bd oxidase (Table 1). Since the activity of bd oxidase is lower compared to that of bo3 oxidase [1,8] and as the susceptibility to KCN inhibition indicates enzymatic activity mainly of the bo3 oxidase, we conclude that the production of a bd oxidase assembly apparently suppresses the production of the bo3 oxidase. Thus, the cellular levels of bo3 oxidase are higher in the strain carrying the empty plasmid than in the strain producing the WT bd oxidase. Conclusively, the cellular levels of bo3 oxidase are substantially higher in the strains producing the Q_short and Q_heelix variants. However, the amount of bo3 oxidase is significantly reduced in the strain producing the Q_Geo variant to approximately the same level as in the strain producing the WT bd oxidase (Table 1). This suggests that the Q_Geo variant is produced most efficiently among the variants, although it is inactive (Table 1).

The amount of the bd oxidase variants in the mutant membranes was detected by western blot analysis using an antibody raised against the His-tag on CydA (Fig. 4). A band with an apparent molecular mass of about 53 kDa was detected in membranes from wild-type cells (Fig. 4). The molecular mass of CydA as deduced from its DNA sequence is 59 kDa. However, it is well known that due to its hydrophobic nature, CydA binds more SDS than a standard protein resulting in significantly lower apparent molecular masses after SDS/PAGE [12]. The host strain CBO does not encode the bd oxidase, and, consequently, no signal was seen in this lane. Surprisingly, no signal could be detected in membranes from the strain producing the Q_short variant (Fig. 4), indicating that the variant protein is rapidly degraded within this strain. A faint band at around 35 kDa was detectable in the
lane loaded with membranes from the strain containing the Q_helix variant in agreement with its predicted molecular mass of 46 kDa. Thus, the N-terminal helix of the Q-loop indeed stabilizes CydA. A more intense band at 40 kDa was detected in membranes of strain Q_Geo, which contains CydA with a molecular mass of 51.5 kDa. As judged from the intensities, the concentration of CydA in the membrane is about one-third in the Q_Geo mutant and one-tenth in the Q_helix mutant, respectively, in comparison with the wild-type strain. Thus, as expected from activity measurements, the amount of bd oxidase is somewhat reduced in strain Q_Geo, drastically reduced in strain Q_helix, and not detectable in strain Q_short.

Attempts to purify the bd oxidase variants from the mutant strains failed. Membrane proteins were extracted with 1% LMNG, and UV/Vis difference spectra were taken from the cleared extract (Fig. 3). The typical absorbance of the heme groups from bd oxidase was detectable in the extract from the strain producing WT bd oxidase. However, the detergent extract from membranes of the strains expressing the bd oxidase variants showed just the signals from heme b558 at 430, 531, and 562 nm, while signals from heme b595 and heme d were not detectable (Fig. 3). Thus, none of the mutants coding variants of bd oxidase produced a stable and fully assembled bd oxidase. The heme b signals in the extract from the strain containing the empty plasmid most likely derived from bo3 oxidase [8] and succinate dehydrogenase [22]. In an attempt to purify bd oxidase from strain Q_Geo exhibiting the highest amount of the variant oxidase among all mutant strains (Fig. 4), the detergent extract was loaded onto the affinity chromatography column. However, no protein could be eluted from the column, while the flow-through exhibited a UV/Vis spectrum reminiscent to the one obtained with the extract from this strain (Fig. S1). A minor peak within the elution profile most likely derives from bound bo3 oxidase that contains a natural His-tag.

**Conclusion**

The role of the different lengths of the Q-loop of bd-I oxidases from various species is not understood [1,8,22,23]. It might play a functional role in quinol binding and reduction, a structural role in conferring rigidity to the protein, or a combination of both. All bd-I oxidases contain the N-terminal part of the Q-loop that was shown to participate in quinone binding [9,10,24–27]. Furthermore, it builds a hydrophilic loop in the E. coli enzyme that covers heme b558 to protect it from being solvent-exposed [15,16]. Hydrogen/deuterium exchange mass spectrometry (HDX-MS) showed that binding of a quinone-competitive inhibitor just affects the N-terminal part of the E. coli long Q-loop [15]. Members of the L-subfamily of bd-I oxidases feature a C-terminal extension of the Q-loop that is rigid and covers the periplasmic surface of CydA to the interface toward CydB [15,16]. This part...
Fig. 3. UV/Vis dithionite-reduced minus air-oxidized difference spectrum of membranes (A, B) and a detergent extract (C, D) from the parental strain CBO/pET28b(+) (green), strain CBO/pET28b(+) cydA_{his}BX producing the WT bd oxidase (black), and the mutants strains pET28b(+) cydA_{his}BX Q_{short} (orange), pET28b(+) cydA_{his}BX Q_{helix} (blue), and pET28b(+) cydA_{his}BX Q_{geo red} (red). (A, C) show the signals in the Soret region and (B, D) that of the \( \alpha \) - and \( \beta \)-bands. The difference spectra of the mutant strains lack the absorbance of heme d at 628 and 651 nm as well as the absorbance of heme b_{565}. Spectra were normalized to the protein content.
of the Q-loop was not influenced by binding of a quinone and a quinone-competitive inhibitor as determined by HDX-MS [15]. The structures of bd-I oxidase from G. thermodenitrificans and E. coli thus imply that the N-terminal part of the Q-loop is involved in quinone binding, while the C-terminal extension confers stability [14–16]. Here, we show that the deletion of the entire E. coli bd-I oxidase Q-loop either led to a perturbed production or a rapid degradation of the oxidase so that no signal from CydA was obtained in western blot analysis. Retaining solely the N-terminal helix of the Q-loop led to the production of an inactive enzyme that is not fully assembled (Figs 3 and 4, Table 1). The same phenotype was observed when the long Q-loop was replaced by the short one from the G. thermodenitrificans enzyme in the Q_{Geo} variant or, in other words, when the rigid C-terminal part of the Q-loop was lacking (Fig. 1). Note-worthy, this protein accumulated in considerable amounts in the mutant membranes (Fig. 4). However, the Q_{Geo} variant seems to be too fragile to withstand protein purification (Figs 3 and S1). These findings support the proposed structural role of the C-terminal extension that might be caused by the special mechanistic needs of the E. coli enzyme. Because the long Q-loop extends to the CydAB interface [15,16], it might strengthen the interactions between the two subunits. The oxygen entry channel of E. coli bd-I oxidase starts on CydB and extends further to heme d in CydA. A close connection between the two subunits might be necessary to maintain this substrate channel. This is in agreement with the fact that homologues of CydY have so far only been detected in bd-I oxidases from the L-subfamily [15] and suggests that all enzymes belonging to this subfamily employ CydY to seal the common oxygen entry site, and thus, have to use the alternate oxygen access channel. It is most likely that the acquisition of CydY led to the evolution of the extended oxygen entry channel, a necessity to maintain a functional enzyme. Accordingly, all oxidases featuring a homologue of CydY would share these properties. Following this line of arguments, the presence of CydY should be concomitant with a long Q-loop and both indicators might be used as a probe to classify oxidases as functioning mainly as terminal oxidases exemplified by the E. coli bd oxidase or detoxifying enzymes exemplified by the G. thermodenitrificans enzyme [16].

Table 1. NADH oxidase activity of membranes from strains producing variants of bd oxidase or lacking both bd oxidases. All measurements were performed as triplicates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific NADH oxidase activity</th>
<th>Specific NADH oxidase activity</th>
<th>Inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(- KCN) (U·mg^{-1})</td>
<td>(+ KCN) (U·mg^{-1})</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>0.81 ± 0.002</td>
<td>0.05 ± 0.001</td>
<td>94</td>
</tr>
<tr>
<td>cydA_{hisBX}</td>
<td>0.42 ± 0.001</td>
<td>0.26 ± 0.001</td>
<td>38</td>
</tr>
<tr>
<td>cydA_{hisBX} Q_{short}</td>
<td>0.64 ± 0.001</td>
<td>0.04 ± 0.002</td>
<td>94</td>
</tr>
<tr>
<td>cydA_{hisBX} Q_{helix}</td>
<td>0.68 ± 0.002</td>
<td>0.04 ± 0.001</td>
<td>94</td>
</tr>
<tr>
<td>cydA_{hisBX} Q_{Geo}</td>
<td>0.40 ± 0.002</td>
<td>0.03 ± 0.001</td>
<td>93</td>
</tr>
</tbody>
</table>

Fig. 4. Western blot of membranes from E. coli strains CBO/pET28b(+) cydA_{hisBX} (WT), CBO/pET28b(+) (CBO), CBO/ pET28b(+) cydA_{hisBX} Q_{short} (Q_{short}), CBO/ pET28b(+) cydA_{hisBX} Q_{helix} (Q_{helix}), and CBO/pET28b(+) cydA_{hisBX} Q_{Geo} (Q_{Geo}). Each lane was loaded with 100 µg membrane protein. CydA was detected with an antibody raised against the His-tag. The apparent molecular masses of the marker proteins (lane M) are given on the left side.
Acknowledgements

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Author contributions

AT grew the cells, determined the activity, isolated membranes and the protein, and recorded the spectra and made the western blot; SB made the mutants; DW made Fig. 1; TF designed research and wrote the manuscript with input from all authors.

References


**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Attempts to purify the *Q*<sub>Geo</sub> variant by affinity chromatography.

**Table S1.** Amino acid sequence of the Q-loop present in *Q*<sub>short</sub>, *Q*<sub>helix</sub> and *Q*<sub>Geo</sub>.

**Table S2.** Sequence of pEX-A128.

**Table S3.** Oligonucleotides used in this work.

**Table S4.** Plasmids used in this work.