An Unconventional Copper Protein Required for Cytochrome c Oxidase Respiratory Function under Extreme Acidic Conditions

Very little is known about the processes used by acidophile organisms to preserve stability and function of respiratory pathways. Here, we reveal a potential strategy of these organisms for protecting and keeping functional key enzymes under extreme conditions. Using Acidithiobacillus ferrooxidans, we have identified a protein belonging to a new cupredoxin subfamily, AcoP, for “acidophile CcO partner,” which is required for the cytochrome c oxidase (CcO) function. We show that it is a multifunctional copper protein with at least two roles as follows: (i) as a chaperone-like protein involved in the protection of the CuA center of the CcO complex and (ii) as a linker between the periplasmic cytochrome c and the inner membrane cytochrome c oxidase. It could represent an interesting model for investigating the multifunctionality of proteins known to be crucial in pathways of energy metabolism.

Bacteria that can oxidize Fe(II) inhabit many environments where Fe(II) is present. One of these is Acidithiobacillus ferrooxidans, a Gram-negative strictly acidophilic bacterium. It has become an interesting field of research both for biology, as a model organism for studying life in an extremely acidic environment, and for engineering, because of its use in microbial leaching. Its respiratory chain has been analyzed in detail in recent years. Bioinformatic analysis of its genome sequence has allowed the identification of the main components of the electron transport chain involved in iron oxidation (1). Most of these components, i.e. two c-type cytochromes (Cyc1 and Cyc2), subunits of the aa3-cytochrome c oxidase (CoxBACD), rusticyanin (RcY), and a protein with unknown function (Orf1), are encoded by the rus operon (2). With the exception of Orf1, they have been well characterized and their functions recognized (3–7). After purification in mild conditions, these proteins interact with each other, forming a supercomplex, also called a respirasome, that spans the outer and the inner membranes and transfers electrons from iron to oxygen (8). Orf1 is present in this supercomplex, raising the question of its role in such a complex and in the pathway. Unfortunately, no efficient genetic systems in A. ferrooxidans are available for disrupting genes by procedures such as homologous recombination for investigating the physiological role of Orf1, and comparison with other known cytochrome c oxidase systems does not permit elucidation of the function of Orf1. To characterize it and to shed light on its physiological role, we have combined activity measurements, spectroscopic analysis, and SPR experiments, which revealed that it is a novel type of copper-binding protein. We have called it AcoP (for “acidophile CcO partner”). We propose that it is important for the stability and activity of CcO under acidic conditions. It also seems to act as a bridge that allows the contact between components of the respiratory chain illustrating a dual role of AcoP in this machinery.

EXPERIMENTAL PROCEDURES

Protein Purification

Cytochrome Cyc1 and RcY were purified as described previously (8). For purification of cytochrome c oxidase and AcoP from A. ferrooxidans, spheroplasts were done as described previously (9, 10). Membrane solubilization was done using dodecyl-β-D-maltoside at 1.5 mg/mg of protein. The suspension was ultracentrifuged at 200,000 × g for 1 h, and the resulting supernatant was concentrated and loaded onto a 10–30% sucrose density gradient in 50 mM sodium acetate (pH 5), 5% glycerol, 0.01% dodecyl-β-D-maltoside, and 0.05% aminocaproic acid (buffer A). After dialysis, the fraction containing cytochrome c oxidase (recovered at 23–25%) was loaded onto a DEAE-cellulose column equilibrated in buffer A. The flow-through containing aa3-CcO was loaded onto a hydroxylapatite column equilibrated with 50 mM sodium acetate (pH 5.3), 5% glycerol, 0.01% dodecyl-β-D-maltoside, 0.05% aminocaproic acid. The complex containing aa3-cytochrome c oxidase activity was eluted by applying 1 M phosphate (pH 5.3) to the column.

Complexes with various amounts of AcoP were obtained by loading the 1 M phosphate fraction after subsequent dialysis into buffer A onto a Mono S column (FPLC apparatus, GE Healthcare) equilibrated with buffer A, at a flow rate of 1 ml/min. Complexes were eluted by a linear gradient from 0 to 500 mM NaCl. Cytochrome c oxidase depleted of AcoP was found in the flow-through, whereas fractions of CcO containing a low or high amount of AcoP was eluted at 200 and 300 mM NaCl, respectively. These three different fractions presenting
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specific spectroscopic signals of cytochrome c oxidase were concentrated, and proteins were identified by N-terminal sequencing.

AcoP from A. ferrooxidans was obtained as a by-product of the CcO purification during the step of the hydroxylapatite column at 500 mM phosphate (pH 5.3) or by heterologous expression (8).

Analytical Procedures

Electrophoresis—Proteins were loaded on 4% polyacrylamide stacking, 10% running SDS, or native gels or 5–15% Blue Native gels according to Ref. 11 (MiniProtean3 apparatus, Bio-Rad). After migration, proteins were revealed with Coomassie Blue, 3,3',5,5'-tetramethylbenzidine, specific for hemes (12), specific staining for cytochrome c oxidase, and for bc complex or antibodies.

Western Blots—Proteins separated on denaturing or native gels were transferred to nitrocellulose membrane (Electran) with the Fast blot apparatus (Biometra) at 5 mA/cm² for 45 min. gels were transferred to nitrocellulose membrane (Electran) antibodies.

Activity Measurements

CcO activity with reduced cytochromes c (Cyc1 or cytochrome c from bovine heart) as electron donors was measured in 50 mM sodium acetate (pH 5) at room temperature and under atmospheric oxygen. Cytochromes were previously reduced by ascorbate, and the excess ascorbate was removed on a PD10 column. Oxidation of 50 μM reduced cytochrome c from bovine heart or 5 μM reduced Cyc1 or 5 μM reduced N,N,N',N'-tetramethyl-p-phenylenediamine was monitored at 552 or 562 nm (for N,N,N',N'-tetramethyl-p-phenylenediamine oxidase activity) after addition of 0.23 μM CcO-AcoP⁺, CcO-AcoP⁻, and CcO-AcoP⁻, all stored in buffer A. One unit of cytochrome c oxidase activity corresponds to the consumption of 1 nmol of oxidized cytochrome c per min. When mentioned, exogenous AcoP (1.1 μM) was added to the three different fractions, and CcO activity was subsequently measured (10 min after AcoP addition) as described above.

Experiments under physiological acidic pH correspond to dialysis of a fraction of the cytochrome c oxidase (CcO-AcoP⁺ or CcO-AcoP⁻) against 50 mM glycine at pH 2. Subsequently, cytochrome c oxidase activity measurements were performed under the usual conditions described above. In some cases, after pH dialysis of CcO-AcoP⁺, the enzyme was incubated with an excess of exogenous AcoP (1.1 μM); in other cases, an excess of exogenous AcoP (1.1 μM) was added to CcO prior to buffer exchange, and the distinction between the two cases is clearly mentioned in the text. In-gel CcO detection was done on a blue-native gel according to Ref. 8.

SPR Binding Experiments

The interaction between AcoP and Cyc1, RcY, or CcO-AcoP⁻ was investigated with an analytical system based on a biomolecular interaction biosensor (BIAcore T100). AcoP was immobilized on a CM5 sensor chip (BIAcore) in 50 mM Hepes buffer, 150 mM NaCl, 0.05% Tween 20 (pH 7.4) by amine coupling using a kit supplied by BIAcore. All experiments were subsequently done in 100 mM ammonium acetate, 100 mM NaCl, 0.05% Tween 20 (pH 5.6). Global fitting of the exponential curves (sensorgrams) giving both kₘₐₜ and kₜₜₜ values were performed using the BIAeval software. Control experiments were done on sensor chip in the absence of immobilized protein.

Mass Spectrometry

Tryptic digestion of excised gel plugs, MALDI-TOF, and ion trap LCQ-DECA XP mass spectrometry protein identification were performed as described previously (8).

EPR Spectroscopy

EPR spectra were recorded at liquid helium temperatures with a Bruker ESP 300e X-band spectrometer fitted with an Oxford Instruments cryostat and temperature control system. The instrument setting was microwave frequency at 9.47 GHz.

Sequence Alignments and Structural Modeling

Sequences were retrieved from the NCBI server (www.ncbi.nlm.nih.gov) and aligned with Clustal W (13). CoxB was modeled using Modeler software (14–16) with Cox2 from Pseudomonas denitrificans as template (17). The resulting theoretical model was displayed and analyzed with PyMOL software.

RESULTS

Identification of a New Subfamily of Cupredoxin—A PSI-Blast search using the amino acid sequence of AcoP identified homologues with E-values below 10⁻¹⁰ belonging to the cupredoxin superfamily. There are few sequence identity of the proteins in this superfamily is low, but they all have a conserved β-barrel fold, the so-called cupredoxin fold, and are defined as copper-containing proteins that play central roles in several crucial cellular processes (18). Secondary structure prediction of AcoP confirms that it belongs to the cupredoxin family. Despite the low sequence similarity, there is substantial identity in the immediate vicinity of the canonical copper ligands in the C-terminal part of AcoP, i.e. Cys-159, His-166, and Met-171 or His-172 (supplemental Fig. S1A). The secondary prediction shows that Met-171 and His-172 are not present in the last β-sheet, and the two residues could be involved in copper ligation. The 4th ligand varies among cupredoxins and consequently is the most difficult ligand to assign; His-85 as well as Asp-59 might be potential candidates (supplemental material).
We can conclude from this first analysis that AcoP belongs to the wide family of cupredoxins. However, its function could not be assigned by sequence analysis. Interestingly, a comparative genomic analysis reveals that other genomes of the *Thiobacillus* family have a similar organization with the acoP gene located upstream of genes encoding for CcO (supplemental Fig. S1B) (2). This suggests that the function of AcoP may be closely related to CcO. Intriguingly, the system-specific accessory proteins (such as Sco1, Cox17, and Cox11) described as crucial for a correct maturation of CcO in most living organisms (19, 20) are not found in these bacteria (supplemental Fig. S1B).

**Stable Association of AcoP and CcO**—Our previous studies have shown that AcoP is present in the iron respiration supercomplex composed of periplasmic, inner, and external membrane proteins (8). However, the cellular localization of AcoP in the supercomplex was still unknown. Use of various prediction programs did not allow us to determine it; either a transmembrane helix or a signal peptide is predicted. To assay the strength of the membrane association of AcoP, membrane samples were treated with chaotropic reactants. The persistence of AcoP on the membrane fraction, even after drastic washing, indicates that it is either a membrane protein or it interacts very tightly with a membrane protein of the supercomplex, such as the CcO (supplemental Fig. S2). To define the potential relation between CcO and AcoP more precisely, we purified CcO from spheroplasts under conditions that destabilize the supercomplex. The isolated sub-complex with CcO activity shows a single band on Blue Native (BN) PAGE (Fig. 1A), a technique commonly used to preserve native complexes while still keeping the migration size-dependent. The composition of this band was analyzed by ion-trap mass spectrometry (supplemental Table S1) as well as by SDS-PAGE coupled to MALDI-TOF mass spectrometry (Fig. 1B) and confirmed by Western blotting (Fig. 1C). These experiments revealed that AcoP copurified with CcO, which has been identified by the presence of the two canonical subunits of typical *aa*₃-type CcO (CoxA and CoxB). CoxC and CoxD were not detected, probably due to their high levels of hydrophobicity. These results show that AcoP is tightly associated to CcO and forms a stable complex CcO-AcoP.

**Unconventional EPR Signal Observed in CcO Samples**—The likelihood that AcoP might be a copper-binding protein led us to search for further evidence by EPR. The UV-visible spectrum of CcO samples containing AcoP is dominated by the typical absorption peaks of *aa*₃-type oxidases at 597 and 440 nm corresponding to the *a*-type hemes present in CoxA subunit (data not shown). Therefore, EPR was performed on the CcO-AcoP sample and recorded on its oxidized form. As expected, the spectrum of the CcO-AcoP complex contains typical CcO features of low spin heme *a* and of the dinuclear copper center Cuₐ from the CoxB subunit (in the field region between 260 and 330 mT (Fig. 2A, line 1)). Unexpectedly, however, a strong signal is present at *g* = 2 that is not observed in regular *aa*₃-type CcO. Running the *g* = 2 region of the spectrum at lower modulation amplitudes, *i.e.* increasing the field resolution, reveals a complex multiline spectrum centered at *g* = 2. Power and temper-
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ature variation demonstrate that this spectral feature is strongly saturated under the conditions applied to run the spectrum shown in Fig. 2A (optimized for the observation of low spin hemes). An unsaturated spectrum was obtained above 120 K (Fig. 2B, line 3), which does not arise from any of the standard metal-based paramagnetic centers but is at the same time too wide in spectral range to correspond to an organic radical.

Purified AcoP yields a visible spectrum dominated by an adsorption peak at 620 nm (supplemental Fig. S3A) and an EPR spectrum similar to the g = 2 part of the CcO-AcoP complex at 120 K (Fig. 2, A and B, lines 2 and 4), so the peculiar paramagnetic center is harbored by AcoP and not by one of the subunits of standard aa₃-oxidase.

EPR spectra of methane monooxygenase (pMMO), a multicyanoc protein isolated from Methylosinus trichosporium, have similar features (21). Although the structural properties of the paramagnetic center could not be identified, there was an almost perfect point-symmetric structure of the spectrum (with regard to g = 2), and the authors proposed that this feature is reminiscent of at least a spin-polarized doublet species. We therefore hypothesize that rather than being due to a mononuclear copper ion, this paramagnetic species observed for AcoP arises from coupled copper atoms. ICP-MS metal analysis confirmed this and revealed the presence of copper in purified AcoP fractions with about two copper atoms per AcoP. Studies on CcO-AcoP fractions further corroborated these findings because they revealed the presence of more than five copperpeaks per complex, three of which must belong to the Cu₄ and Cu₅ centers of CcO.

AcoP Is Essential for Optimal CcO Activity—To examine the precise role of AcoP in CcO, an additional purification column was used to separate these two partners. SDS-PAGE analysis on three different fractions followed by mass spectrometric analysis and Western blot shows that the only difference between these fractions is the amount of AcoP (Fig. 3, A and B). This allowed us to define three kinds of CcO samples as a function of the amount of AcoP as follows: (i) the enzyme without AcoP (CcO-AcoP⁻), (ii) containing low (CcO-AcoP⁻), or (iii) large (CcO-AcoP⁺) amounts of AcoP.

BN gels were run with these three different samples and stained in-gel for CcO activity. They showed the following: (i) a homogenous electrophoretic pattern, suggesting that AcoP is not directly involved in the quaternary structure of CcO even if a slight difference in size is observed (Fig. 3C), and (ii) a strong correlation between the decrease of CcO activity and the amount of AcoP present in the complex (Fig. 3D). The same trend was observed with N,N,N',N'-tetramethyl-p-phenylenediamine, a chemical reagent used in the assay as electron donor instead of the cytochrome c. This confirms that the AcoP effect observed in this experiment is CcO-dependent and cytochrome c-independent. Specific activity assays show that CcO-AcoP⁻ presents no activity in contrast to CcO-AcoP⁺ (Fig. 3E) and to CcO-AcoP⁻ which shows intermediate levels of CcO activity. As a control, CcO activity was measured on AcoP alone, which does not present any activity (data not shown). Overall, this experiment clearly highlights that AcoP is essential to preserve an optimal CcO activity.

In line with the results obtained above, the EPR spectrum of the CcO-AcoP⁻ sample does not show the atypical signal centered at g = 2 at 120 K (supplemental Fig. S3B, line 2). A decrease of the Cu₄ signal as well as a more heterogeneous and smeared out spectrum is observed suggesting either a modification in the direct environment of or, more simply, a loss of Cu₄ (Fig. 4). Copper quantification via ICP-MS analysis supports the latter hypothesis as only one copper/CcO is present in this sample, most likely the copper from the Cu₅ center. These results suggest that AcoP maintains an optimal oxidase activity by protection against copper depletion of the redox active center.

When excess of purified AcoP is added to CcO-AcoP⁻ and CcO-AcoP⁺, the CcO activity is partially reactivated (Fig. 3F). As the full activity is not recovered in either case as compared with CcO-AcoP⁺, we postulate that part of the enzyme devoid of AcoP might have been irreversibly inactivated. This suggests that AcoP is indeed critical for maintaining the CcO in an optimal and stable form and that part of inactive CcO can be reactivated by AcoP.

AcoP Assists CcO under Acidic Physiological Conditions—

The above experiments described were performed at pH 5,
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Although proteins located in the periplasm of *A. ferrooxidans* encounter extreme acidic conditions, close to pH 2, to define the impact of an acidic environment toward CcO, we measured its activity at physiological pH. After CcO incubation at pH 2, there is a decrease of more than 40% in the activity of the CcO-AcoP⁺ sample (Fig. 5A). Subsequent addition of AcoP allows a total recovery of the initial CcO activity (Fig. 5A). A similar trend is also observed for CcO-AcoP⁻ (data not shown). These results suggest that even in the enriched fraction called CcO-AcoP⁺, the required stoichiometry of AcoP with respect to the CcO is probably not yet optimized for full protection of its partner enzyme. This hypothesis is confirmed by SPR experiments showing an interaction between CcO-AcoP⁺ and AcoP (supplemental Fig. S4). Moreover, we have shown that the reactivation of CcO is AcoP-specific, as other acid-stable proteins with a cupredoxin fold, such as RcY, could not induce the reactivation process. To evaluate the ability of AcoP to maintain optimal CcO activity under acidic conditions, CcO was preincubated in the presence of AcoP prior to the pH change. Under these conditions, less than 16% of activity was lost (Fig. 5B). AcoP can thus prevent inactivation of CcO, probably by maintaining an active conformation of the CcO, and furthermore, AcoP can reactivate it over time, as 100% of the activity was recovered after 60 min (Fig. 5B). This demonstrates that AcoP plays a critical role in preventing, as well as actively recovering, partial loss of CcO activity under extreme, albeit physiological, pH conditions.

EPR spectra of CcO-AcoP⁺ were recorded at pH 2 and at 120 K to increase the spectral resolution of the 

**FIGURE 4. Decrease of Cu₄ EPR signal in the absence of AcoP.** CcO-AcoP⁺ (0.23 μM) at pH 5 (100% corresponds to 500 units/mg) after pH change (pH 2) and after subsequent addition of exogenous AcoP (1.1 μM) (pH 2 → AcoPexog⁺). B, prior to pH change, exogenous AcoP (1.1 μM) was added to CcO-AcoP⁻ (this step is represented by "pH₂ + AcoPexog⁻" in the figure). Time 0 corresponds to the buffer exchange to pH 2, and specific activity of CcO was measured at different incubation times.

**FIGURE 5. CcO at physiological pH role of AcoP.** A, specific activity of CcO-AcoP⁺ (0.23 μM) at pH 5 (100% corresponds to 500 units/mg) after pH change (pH 2) and after subsequent addition of exogenous AcoP (1.1 μM) (pH 2 → AcoPexog⁺). B, prior to pH change, exogenous AcoP (1.1 μM) was added to CcO-AcoP⁻ (this step is represented by "pH₂ + AcoPexog⁻" in the figure). Time 0 corresponds to the buffer exchange to pH 2, and specific activity of CcO was measured at different incubation times.

As mentioned previously, AcoP has been identified in the supercomplex involved in iron oxidation pathway. Each protein present in this supercomplex has been purified, and SPR experiments were used to investigate the other potential physiological partners of AcoP. In line with our previous results (8), no interaction was observed between AcoP and RcY (supplemental material), whereas interaction between AcoP and Cyc1 was detected with an apparent *K₅ₐ* of 5.10⁻⁷ M (Fig. 7). Consequently AcoP is not only able to tightly bind the CcO, it can also bind the soluble periplasmic cytochrome Cyc1, underlining an additional role of AcoP as a bridge between these two partners acting like a potential electron wire.

**DISCUSSION**

The function of a previously unknown protein, Orf1, has been elucidated. We first demonstrated the existence of a tight interaction with the CcO, and we called the protein AcoP, for acidophile CcO partner. We then identified it as a cupredoxin protein that appears critical for the stability of CcO and for its optimal function in extremely acidic physiological conditions. We could not, however, assign it to a particular subfamily of cupredoxins, on the basis either of its function or of its
sequence, and consequently we propose that it belongs to a new subfamily. Its EPR spectral features reveal the presence of an unusual paramagnetic species that most likely arises from copper multimerization, a characteristic shared with the pMMO protein (23, 24). As only one copper-binding site is evidenced by the sequence analysis, this observation raised the question as to how the multicopper center would bind to AcoP. An experimental answer to part of this question on the detailed molecular structure of the copper ion(s) giving rise to this EPR spectral species has to await study of the samples by advanced EPR techniques. For the time being we cannot produce samples in the quantities required for these approaches.

We propose several nonexclusive roles of the function of AcoP on the CcO complex as follows. (i) An unambiguous role as a chaperone-like protein can be attributed to AcoP. We define it as a “chaperone-like” protein because, in contrast to typical chaperone proteins, the interaction between AcoP and CcO is not transient (25, 26). Nevertheless, removal of this protein leads to a drastic loss of CcO function. This effect is partially reversible; addition of exogenous AcoP can recover part of the activity of CcO. The presence of AcoP also allows us to maintain a stable and active conformation of CoxB and consequently an optimal CcO activity in extreme acidic physiological conditions. Overall, these results illustrate an unprecedented characterization of a chaperone-like protein of a CcO. This alternative strategy to protect the metal center from acid stress via the presence of a supplementary protein seems to have been developed rather than a modification of the surrounding center itself (27, 28). This type of strategy to protect crucial enzymes of extremophiles from harsh conditions has also been proposed in Thermus thermophilus, wherein an additional protein (Nqo15) has been found to bind tightly to the “core” of complex I (29, 30). As this protein features the fold of the frataxin family (known as iron chaperones) (31), it was proposed to have a potential role as a chaperone-like protein to stabilize complex I and/or as an iron storage for Fe-S cluster regeneration. The presence of this supplementary protein appears to be unique to thermophiles and may be a consequence of adaptation to extreme environments, underlying a potential common mechanism shared with AcoP for acidophiles.

(ii) A second role as an electron wire is also proposed, as AcoP is found in the iron respiratory supercomplex, and it interacts with CcO as well as with the known electron partner of CcO, the soluble cytochrome c Cyc1. There are several reports on the interaction between aa3-CcO and soluble cytochrome c in different organisms (32–34). Two principal steps have been highlighted as follows: first, an electrostatic interaction that allows a pre-orientation of the two partners, and second, a hydrophobic interaction, essential for an efficient electron transfer. Sequence comparisons of several CoxB homologues show conservation of the major residues involved in these two steps (supplemental Fig. S5). The first step described above probably does not occur in A. ferrooxidans, as residues negatively charged at neutral pH become protonated at pH 2, and another mechanism has been implicated, i.e. the presence of a supplementary protein (AcoP) brings Cyc1 and CoxB in close proximity and then allows efficient electron transfer between Cyc1 and CoxB.

This need of adaptation to optimize electron transfer in extreme environments has also been shown in the T. thermophilus CcO.
nopolus system, in which interaction between CcO and soluble cytochrome c involves mainly hydrophobic groups without the usual ionic contacts, probably because the electrostatic attractions might be weakened at elevated temperatures (35, 36).

Our observations illustrate the critical role of AcoP in CuA stability, partner redox interaction, and CcO activity. AcoP emerges as a multifunctional protein essential for oxygen respiration in A. ferrooxidans. Another protein conserved among most of the living organisms and well known as essential for the CcO system is Sco1. The notion of a multifunctional protein is suggested for Sco-type proteins, which may exhibit two or more distinct roles (37, 38). Sco1 has first been described as a metallochaperone involved in copper transfer to the CcO system. More recently, it has been suggested that Sco1 might also behave like a redox protein (37); and a tight association with the fully assembled CcO complex suggests another unknown role than the transient post-translational CcO maturation protein (38). Even if there is no sequence similarity between Sco1 and AcoP, similar multifunctional properties are now demonstrated for both proteins. The presence of AcoP may correspond to an adaptation of A. ferrooxidans to face acidic conditions and could explain the absence of a Sco1-like protein in acidophilic organisms containing AcoP. The hypothesis that the copper of AcoP may serve like Sco1 as a metal storage for the redox active copper center of the cytochrome c oxidase remains to be checked.

The study of AcoP presents two main implications as follows: (i) an important similarity in terms of function with the Sco1 family makes its study of central importance for a large scientific community related to the CcO system; (ii) a chaperone-like role to protect a key protein of the respiratory chain against acidic damage also makes this study significant. Not only acidophilic organisms seem to possess acid-chaperone proteins, but, in addition, bacteria susceptible to acid stress, such as enterobacteria, have developed a similar strategy to protect their periplasmic proteins (39). Consequently, the implications of this study might also have an important impact on the understanding of general protein protection against acid stress.

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