



RESEARCH LETTER

The stress response protein Gls24 is induced by copper and interacts with the CopZ copper chaperone of *Enterococcus hirae*

Jivko V. Stoyanov¹, Stefano Mancini¹, Zen Huat Lu¹, Frédéric Mourlane¹, Kristian R. Poulsen², Reinhard Wimmer³ & Marc Solioz¹

¹Department of Clinical Pharmacology and Visceral Research, University of Berne, Berne, Switzerland; ²Department of Physics and Nanotechnology, Aalborg University, Aalborg, Denmark; and ³Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Aalborg, Denmark

Correspondence: Marc Solioz, Department of Clinical Pharmacology and Visceral Research, University of Berne, Murtenstrasse 35, 3010 Berne, Switzerland. Tel.: +41 31 632 3268; fax: +41 31 632 4997; e-mail: marc.solioz@ikp.unibe.ch

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Introduction

Copper is both an essential and a toxic trace metal in living organisms. It acts as a cofactor for > 30 enzymes, such as superoxide dismutase, cytochrome *c* oxidase or lysyl oxidase, but toxicity can arise when excess copper accumulates in the cell (Linder & Hazegh Azam, 1996). The two oxidation states of copper, Cu⁺ and Cu²⁺, not only allow its participation in essential redox reactions, but also to form reactive oxygen species that are known to cause cellular damage. Hence, maintenance of copper homeostasis in living organisms is critical.

In the Gram-positive bacterium *Enterococcus hirae*, the *cop* operon is a key element in the maintenance of copper homeostasis (Solioz & Stoyanov, 2003). The operon encodes four proteins: two copper ATPases, CopA and CopB, a copper-responsive repressor, CopY, and a copper chaperone, CopZ (Odermatt *et al.*, 1993; Odermatt & Solioz, 1995). CopZ belongs to a family of metallochaperones that are conserved

Abstract

Intracellular copper routing in *Enterococcus hirae* is accomplished by the CopZ copper chaperone. Under copper stress, CopZ donates Cu⁺ to the CopY repressor, thereby releasing its bound zinc and abolishing repressor–DNA interaction. This in turn induces the expression of the *cop* operon, which encodes CopY and CopZ, in addition to two copper ATPases, CopA and CopB. To gain further insight into the function of CopZ, the yeast two-hybrid system was used to screen for proteins interacting with the copper chaperone. This led to the identification of Gls24, a member of a family of stress response proteins. Gls24 is part of an operon containing eight genes. The operon was induced by a range of stress conditions, but most notably by copper. Gls24 was overexpressed and purified, and was shown by surface plasmon resonance analysis to also interact with CopZ *in vitro*. Circular dichroism measurements revealed that Gls24 is partially unstructured. The current findings establish a novel link between Gls24 and copper homeostasis.

from bacteria to humans (Harrison *et al.*, 2000). Under conditions of copper excess, CopZ donates Cu⁺ to the CopY repressor. This leads to the replacement of the Zn²⁺ cofactor of CopY by two Cu⁺ ions and a concomitant decrease in DNA affinity, which in turn induces the expression of the *cop* operon (Strausak & Solioz, 1997; Cobine *et al.*, 2002). The transfer of copper from CopZ to CopY involves protein–protein interaction, thereby conferring specificity to the process (Cobine *et al.*, 1999; Portmann *et al.*, 2004). CopZ was also shown to interact with the CopA copper ATPase, the presumed entry point of copper into *E. hirae* (Multhaup *et al.*, 2001).

To identify other intracellular targets of CopZ, we used a yeast two-hybrid screen (Cowell, 1997). Using CopZ as a bait, we identified a new protein interacting with CopZ. We call this protein Gls24, based on the 72% sequence identity it exhibits to the ‘stress-response regulator’ Gls24 of *Enterococcus faecalis* (Giard *et al.*, 1997). CopZ also interacted with Gls24 *in vitro*, as assessed by surface plasmon resonance analysis. Gls24 is encoded by an eight-gene operon,

which is strongly induced by copper. These findings suggest a role for Gls24 in the response of *E. hirae* to copper stress.

Materials and methods

Yeast two-hybrid analysis

Strains and plasmids used for the yeast two-hybrid system were from the Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Palo Alto, CA). Growth and transformation of yeast were performed according to the manufacturer's instructions. The bait plasmid pBZ2 was constructed by excising CopZ (amino acids 16–69) from pDZ69 (Wimmer *et al.*, 1999) with PflmI, followed by Pwo polymerase polishing (Costa & Weiner, 1994) and digestion with PstI. The resultant DNA fragment was ligated into pAS2-1, which had been digested with NcoI/PstI and treated with Klenow DNA polymerase to fill the 5' protruding end of the NcoI site. Plasmid pBZ2 was transformed into *Saccharomyces cerevisiae* Y190, and expression of the fusion protein was verified on a Western blot. A genomic library, consisting of 0.5–1.0-kb *E. hirae* DNA fragments generated by sonication in 50 mM Tris-Cl, pH 8.0, 15 mM MgCl₂, was constructed. DNA fragments were polished with Pwo polymerase, ligated into SmaI-digested, dephosphorylated pACT2, and transformed into *Escherichia coli* XL2-Blue MRF' (Stratagene, La Jolla, CA). Approximately 1.5×10^5 primary clones were amplified by growth for 2 h at 37 °C. These cells were frozen in 25% glycerol at –80 °C for the preparation of plasmid DNA (Humphreys *et al.*, 1975). For screening, the bait plasmid was transformed into Y190, followed by transformation with the genomic library. Transformants were grown at 30 °C for 8 days on minimal medium lacking tryptophan, leucine, and histidine and containing 25 µM of 3-amino triazole. From positive clones, plasmids were isolated and back-transformed into *E. coli*, from where the plasmids were isolated for commercial sequencing (Microsynth, Balgach, Switzerland). The genomic region encoding *gls24* and neighboring genes was derived from a contig of an ongoing genome sequencing project of *E. hirae* ATCC9790 by 454 pyrosequencing. The region had on average 20-fold sequence coverage and was submitted to GenBank (accession number AY927234).

Northern blotting

Cells were grown to the mid-log phase and either not induced or induced with 1 mM CuSO₄ for 1 h. Total RNA was extracted using the RNeasy Midi Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Northern blotting was conducted as described (Sambrook *et al.*, 1989), using 1.2% denaturing agarose gels and Biobond Nylon membranes (Sigma-Aldrich Chemie GmbH, Germany). Signals were detected with a 489-bp

PCR product of the *gls24* gene, obtained with primers fm20 (5'-GCAACTGCAGAGCCCCAGCAAAAGATCC) and fm21 (5'-GAGCTCTCGAGTGCTCAATTGCTGATTGGC) and a 323-bp PCR product of *orf1* obtained with primers sm45 (5'-GTCATCGATCCAGGTCAAAC) and sm46 (5'-ATCGACGGCGATTCATTTCC). PCR fragments were labeled and detected using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Basel, Switzerland) according to the instructions of the manufacturer.

Real-time quantitative PCR

Enterococcus hirae ATCC9790 was grown semi-anaerobically in capped, but not deoxygenated, tubes at 37 °C in M17 medium (Terzaghi & Sandine, 1975). Mid-log cultures were induced as indicated under Results and discussion for 1 h at 37 °C. From 1 mL of culture, RNA was isolated with the Qiagen RNeasy miniprep column kit (Qiagen, Germantown, MD). Quantitative PCR was performed with the QuantiTect SYBR Green I PCR and RT-PCR kits (Qiagen), using 100 ng of RNA per reaction in a total volume of 20 µL in a LightCycler (Roche) and primers js7 (5'-GGTGATGTGACATATGAAGATAAGG) and js8 (5'-CAACATCGACATTGACTTCAATGAC). Cycle conditions were as follows: 45 cycles each of 55 °C for 30 s, 72 °C for 30 s, and 95 °C for 1 s. Expression levels were normalized to 16S rRNA levels.

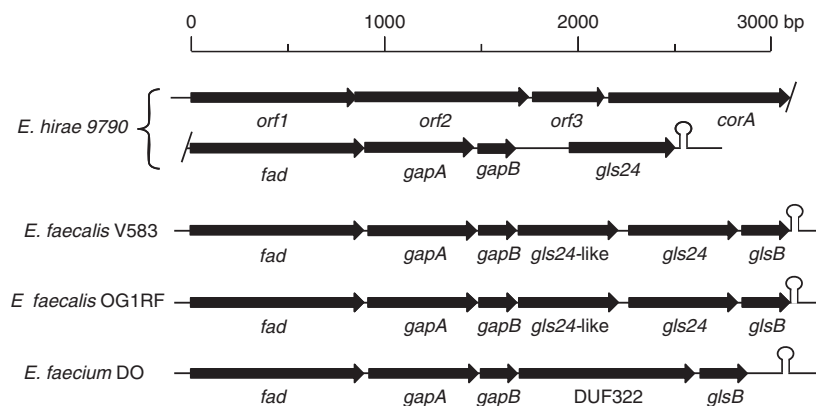
Western blotting

Enterococcus hirae 2-mL cultures in M17 media were grown to an OD_{546 nm} of 0.3–0.5 and induced as described under Results and discussion. Pellets were incubated with 50 µL of 10 mg mL⁻¹ lysozyme in 1 mM EDTA, 10 mM Tris-Cl, pH 8, for 30 min at 25 °C, followed by a freeze–thaw cycle. Ten microliters of 1 mg mL⁻¹ DNaseI in 100 mM MgCl₂ were added and incubation was continued for 10 min at 25 °C. Cell debris was removed by centrifugation for 5 min at 12 000 g. Protein concentrations in the supernatants were determined using the BioRad protein assay (BioRad, Richmond) and 40 µg of protein/lane was used for Western blotting as described (Towbin *et al.*, 1979). Gls24 antiserum was kindly provided by Barbara E. Murray, University of Texas (Teng *et al.*, 2005).

Surface plasmon resonance analysis

The IAsys instrument (Affinity Sensors, Cambridge) was used to measure the binding of CopZ to Gls24. Purified Gls24 was desalted by dialysis against 50 mM Na-HEPES, pH 7.5. A dual-well carboxymethyl dextran cuvette was equilibrated with phosphate-buffered saline, pH 7.4, 0.05% Tween-20, 2% acetonitrile, and 20 µg of Gls24 cross-linked with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. CopZ

Fig. 1. Gene arrangement in *gls24*-encoding operons. The operons of *Enterococcus hirae* (broken across two lines), *Enterococcus faecalis* strains V583 (accession number AE016830) and OG1RF (accession number ABPI01000001.1), and *Enterococcus faecium* DO (accession number AAAK03000003) are shown. The genes are drawn to scale and the position of dyads is indicated by hairpin loops. Genes are described in the text. For simplicity, the same gene names were adopted for all organisms.



was added at 1–10 μM and the interactions were measured at 25 $^{\circ}\text{C}$, with the vibro-stirrer set to 85. Coupling, washing, and calibration steps were performed according to the manufacturer's instructions. The results were evaluated using the GRAFIT software version 5.

Circular dichroism (CD) spectra

CD spectra were recorded on a JASCO J-715 instrument using a quartz cuvette with a light path of 1 mm. The temperature was controlled with a JASCO PTC-348WI Peltier cell. Samples were prepared using 20 μM Gls24 in 10 mM phosphate buffer, adjusted to the desired pH with HCl or NaOH.

Results and discussion

We sought to identify additional targets of CopZ by using the yeast two-hybrid system, using CopZ as a bait. One of two positive clones was subjected to detailed analysis here. The clone contained plasmid pHL7, which encodes the first 40 amino acids of a protein with sequence similarity to Gls24-like proteins; the 40 amino acids of the primary clone apparently represent the CopZ-interacting domain of the protein. Gls24 was originally identified by two-dimensional gel electrophoresis and N-terminal sequencing from *E. faecalis* JH2-2 as a protein induced by glucose starvation (Giard *et al.*, 1997). Similar proteins were later described in *E. faecalis* strains OG1RF, V583, and in *Lactococcus lactis* IL1403 (Capiaux *et al.*, 2000; Giard *et al.*, 2002). A *gls24* deletion strain of *E. faecalis* JH2-2 exhibited a 30% increased doubling time, decreased chain length during growth, and reduced survival of stationary cells in 0.3% bile salts, but there was no significant effect on survival under glucose starvation, 62 $^{\circ}\text{C}$, 20 mM hydrogen peroxide, 0.3 mM CdCl_2 , pH 3.2 or 11.9, and 17% ethanol (Giard *et al.*, 2000). Gls24 was also shown to be involved in the virulence of *E. faecalis* OG1RF (Teng *et al.*, 2005). A strain deleted in *gls24* was considerably less virulent than the wild-type strain in a rat peritonitis model, and an antiserum against Gls24 protected

mice against a lethal challenge of wild-type *E. faecalis*. However, the molecular function of Gls24-like proteins still remains enigmatic.

The genomic region of *E. hirae* encoding the *gls24* gene was obtained from a contig of an ongoing sequencing project in our laboratory. The *gls24* gene appears to be part of an operon containing eight genes and covering a 6-kb DNA region (Fig. 1). This operon thus differs from the *gls24*-encoding operons of the three most closely related, sequenced organisms, namely the *E. faecalis* strains OG1RF and V583, and the *Enterococcus faecium* strain DO, which only feature five or six genes. The first two genes of the *E. hirae* operon, *orf1* and *orf2*, encode proteins with similarity to glycosyl transferases, *orf3* encodes a protein of unknown function, and *corA* encodes a predicted Mg^{2+} transporter. These four genes are unique to the *E. hirae* operon. The following three genes are essentially identical in the four operons depicted in Fig. 1: *fad* encodes a predicted short-chain fatty acid dehydrogenase, *gapA* a trypsin-like serine protease, and *gapB* a protein of unknown function. The remainder of the *E. hirae* operon again exhibits divergence. In *E. faecalis* V583 and OG1RF, the *gapB* gene is followed by a pair of genes that encode proteins with 72% sequence identity. Here, we call these genes *gls24*-like and *gls24*. In contrast, *E. hirae* features a single *gls24* gene, as annotated by manual methods as well as predicted by GLIMMER version 3.02 (Ermolaeva *et al.*, 2001). The *E. hirae* *gls24* gene, which is separated from *gapB* by a 288-bp noncoding region of unknown function, encodes a protein of 179 amino acids with a calculated pI of 4.45 and a molecular weight of 19.7 kDa. It shares 71% sequence identity with Gls24 of *E. faecalis* V583 and OG1RF and is only two amino acids shorter. A different gene arrangement is also found in the operon of *E. faecium* DO. In this organism, a gene encoding a protein with sequence similarity to *gls24*-like proteins, DUF322, takes the place of the *gls24*-like and the *gls24* genes. The founding member of the DUF322 protein family is an alkaline stress response protein of *Staphylococcus aureus* (Kuroda *et al.*, 1995).

All four operons feature the expected -10 and -35 sequence elements and are terminated by stem-loop structures with stabilities of -14 to -26 kcal mol $^{-1}$, which could act as ρ -independent transcription terminators. There is a predicted RNA polymerase σ -factor binding site upstream of *orf1* of the *E. hirae* operon, but no recognition sites for more specific regulatory proteins could be identified using VIRTUAL FOOTPRINT and PRODORIC promoter prediction tools (Munch *et al.*, 2005). In *Pneumococcus*, it was shown that the two-component signal transduction system RR06/HK06 regulates the expression of a *gls24*-like gene (Standish *et al.*, 2007). The RR06/HK06 system regulates numerous genes in *Pneumococcus*, including the major virulence factor choline-binding protein A (CbpA). Currently, it remains unknown to what stimuli the RR06/HK06-system responds, but it is conceivable that a similar system operates in the regulation of the *E. hirae* Gls24-encoding operon.

Gls24 and *gls24*-like genes and the operons encoding them are apparently diverse, even in closely related organisms. The presence of putative glycosyl transferases, proteases, and fatty acid reductases in these operons supports a role in stress response; changes in the fatty acid composition of the membrane and altered cell wall structures are common responses to environmental stress (van de Guchte *et al.*, 2002; Miyoshi *et al.*, 2003; Martinez *et al.*, 2007).

Northern blotting was performed to verify the operon structure of the *E. hirae* *gls24*-encoding region. The same 6-kb mRNA species was detected with probes against *orf1* and *gls24*, supporting the proposed operon structure (Fig. 2a). Expression in control cultures was low, but was markedly induced by copper. A minor band at 5 kb is probably due to mRNA degradation. To assess the induction of *gls24* in quantitative terms, real-time quantitative PCR was performed (Fig. 2b). As reported for other Gls24-like proteins, *E. hirae* Gls24 was induced by glucose starvation, but also by copper and zinc, as well as by oxidative stress induced with paraquat. No induction was observed with the divalent ion chelator *o*-phenanthroline. These results confirm the nature of Gls24 as a stress response protein, but also add copper, zinc, and paraquat as stress signals that induce Gls24.

To confirm induction of Gls24 at the protein level, expression was analyzed by Western blotting, using an antibody against Gls24 of *E. faecalis* OG1RF. Mid-log cells showed low Gls24 expression, which was most strongly stimulated by 1 mM copper (Fig. 3). Glucose starvation and *o*-phenanthroline and, to a lesser extent, zinc and paraquat also stimulated Gls24 expression. These results agree qualitatively with the real-time PCR data, although the induction by *o*-phenanthroline was unexpectedly high. As observed previously by others, the protein band corresponding to Gls24 runs at an apparent molecular weight of 24 kDa (hence the name of the protein; Giard *et al.*, 1997), rather

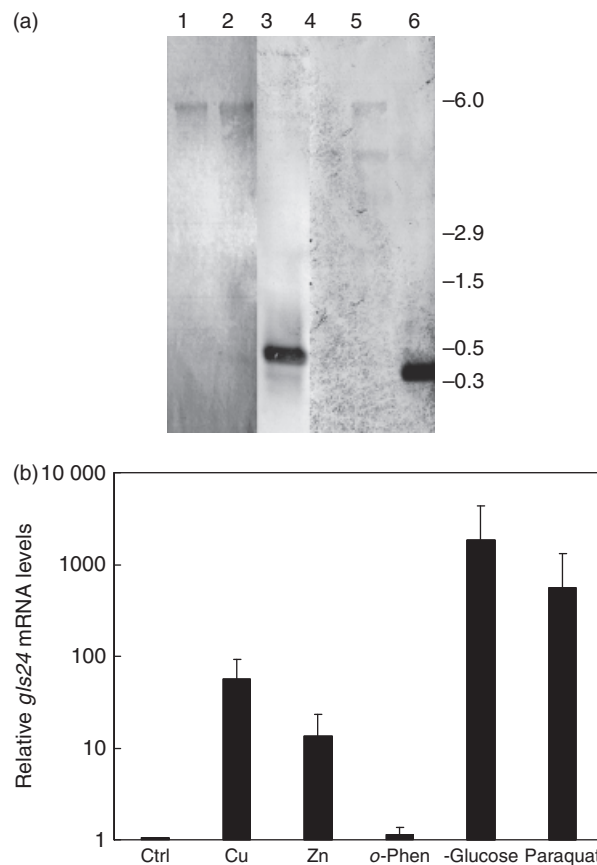


Fig. 2. Gls24 mRNA analysis. (a) Northern blot of *gls24* mRNA. Two micrograms of RNA from uninduced cells (lanes 1 and 4) or cells incubated with 1 mM copper for 1 h (lanes 2 and 5) were probed with a *gls24* probe (lanes 1–3) or an *orf1* probe (lanes 4–6) as described under Materials and methods. As positive controls, lanes 3 and 6 contained 1 ng each of a 0.5-kb fragment of the *gls24* gene or a 0.3-kb DNA fragment of *orf1*. RNA sizes in kilobases are indicated on the right-hand side of the figure. (b) Real-time quantitative PCR analysis of *gls24* mRNA expression. RNA was isolated from cells grown semi-anaerobically and induced for 1 h under the stress conditions indicated on the abscissa. The following conditions were used: no addition (Ctrl), 1 mM CuSO $_4$ (Cu), 100 mM ZnSO $_4$ (Zn), 100 mM *o*-phenanthroline (*o*-Phen), glucose starvation (– Glucose), or 100 mM paraquat (Paraquat). *gls24* mRNA levels relative to the untreated control (Ctrl) are shown. cDNA synthesis and real-time quantitative PCR were conducted as described under Materials and methods. SDs derived from three independent experiments are shown. All differences are significant ($P < 0.05$).

than at the predicted molecular weight of 20 kDa. This could be due to the partially unfolded structure of Gls24.

To study Gls24 *in vitro*, a His-tagged construct of Gls24 was expressed in *E. coli* and purified by Ni-NTA agarose affinity chromatography (not shown). CD was used to assess the folding state of Gls24 and its response to temperature (Fig. 4). The purified protein exhibited approximately 25% α -helix, 25% β -sheet, 25% turn, and 25% random coil. There was no significant change in the CD spectra between

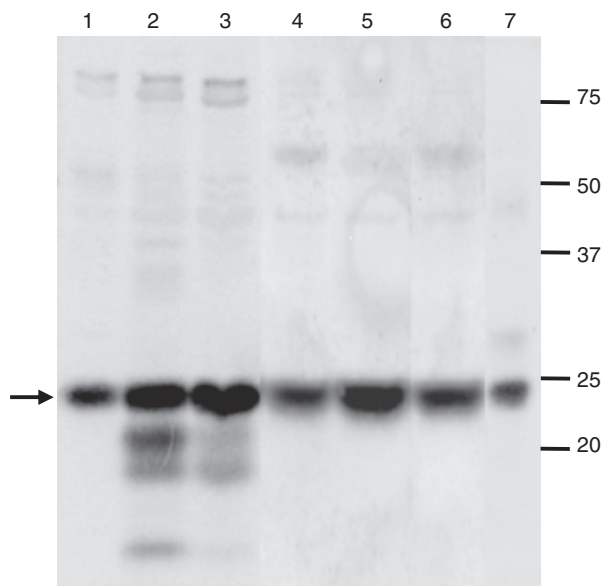


Fig. 3. Western blot of Gls24. Crude extracts of *Enterococcus hirae* were separated by polyacrylamide gel electrophoresis, followed by Western blotting and development with a rabbit anti-Gls24 antibody. Lane 1, uninduced; lane 2, glucose-starved; lane 3, induced with 1 mM CuSO_4 ; lane 4, induced with 0.1 mM ZnSO_4 ; lane 5, induced with 0.1 mM *o*-phenanthroline; lane 6, induced with 0.1 mM paraquat; lane 7, 3 ng of purified Gls24. Molecular weights in kilodaltons are indicated to the right of the blot and the arrow indicates the band corresponding to Gls24.

pH 6.4 and 10. Upon cooling, about 10% of the signals were lost, indicating cold sensitivity of Gls24. The heat denaturation curve showed a broad transition from around 35 to 95 °C and a melting temperature, T_m , of approximately 55 °C. These findings are in line with significant unstructured domains.

To also demonstrate CopZ–Gls24 interaction *in vitro*, surface plasmon resonance was used. Purified Gls24 with the His6-tag cleaved with AcTEV protease was linked to the sensor chip. Gls24 showed a pronounced interaction with CopZ (Fig. 5a). The Gls24– Cu^+ –CopZ interaction could be fitted by single association kinetics according to $R_t = R_{\text{eq}}(1 - \exp^{-k_{\text{on}}t}) + \text{offset}$, where R_t is the instrument response at time t , R_{eq} the equilibrium response, and k_{on} the apparent on-rate at a given CopZ concentration. The offset term allows for differences in the bulk refractive index of the buffers. Figure 5b shows the kinetic plot of k_{on} vs. CopZ concentration. From the slope and the intercept, the following kinetic parameters were derived: $k_a = (1.1 \pm 0.2) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and $k_d = (8 \pm 1) \times 10^{-2} \text{ s}^{-1}$. The resultant K_D for the CopZ–Gls24 interaction was $(7.5 \pm 0.4) \times 10^{-6} \text{ M}$. Thus, CopZ interacted more strongly with Gls24 than with the CopY repressor or the CopA copper ATPase (Multhaup *et al.*, 2001; Portmann *et al.*, 2004). To rule out a nonspecific,

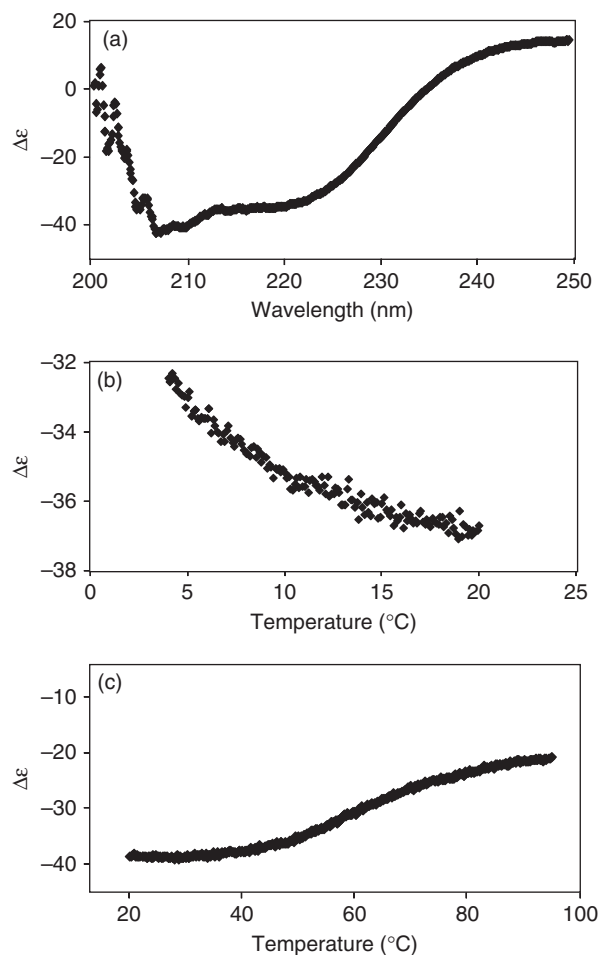


Fig. 4. CD spectra of purified Gls24. (a) Wavelength scan with 20 μM of Gls24 at 20 °C and pH 7.5; (b, c), temperature scans at 220 nm under the same conditions as in (a).

ionic interaction between Gls24 ($\text{pI} = 4.45$) and CopZ ($\text{pI} = 8.52$), lysozyme ($\text{pI} = 9.23$) was included as a control. There was no detectable interaction between Gls24 and lysozyme.

Clearly, the induction of Gls24 by copper and the physical interaction of CopZ and Gls24 *in vivo* and *in vitro* strongly suggest a role of Gls24 in the defense against copper stress in *E. hirae*. Unfortunately, a *gls24* knockout mutant could not be obtained, in spite of several attempts using two different methods. *Gls24* deletion mutants could, however, be generated in *E. faecalis* strains JH2-2 and OG1RF. Both of these organisms harbor two *gls24*-like genes in tandem, while *E. hirae* features only a single *gls24* gene in the operon. Conceivably, inactivating the single *gls24* gene of *E. hirae* is a lethal event.

Copper binds to CopZ in a solvent-exposed position (Huffman & O'Halloran, 2001) and Cu^+ –CopZ could participate in a Fenton-type reaction that generates toxic

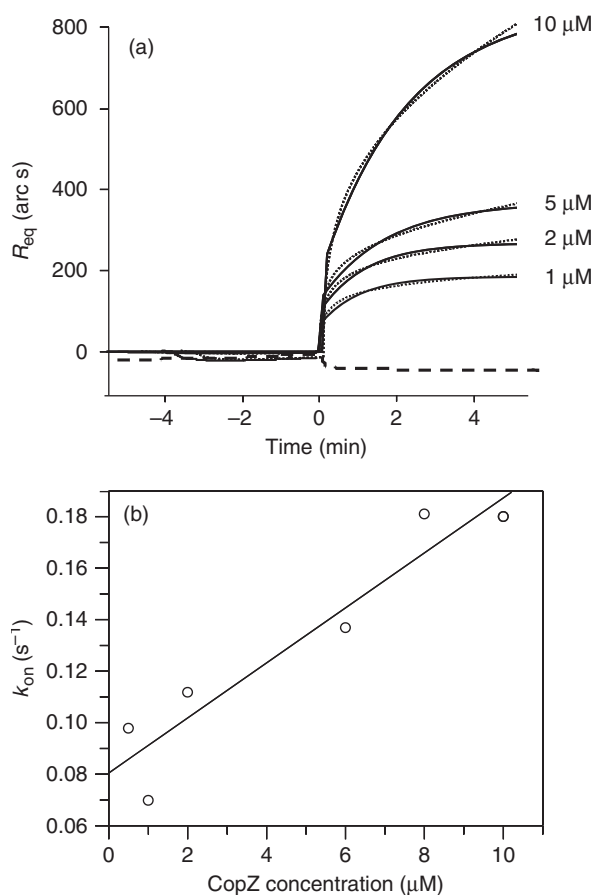


Fig. 5. Surface plasmon resonance analysis of the CopZ–Gls24 interaction. (a) CopZ at the concentrations indicated was reacted with immobilized Gls24 and the association rates were monitored (solid lines). The dotted lines show the fitted single association kinetics and the dashed line shows a control with 10 μ M lysozyme. For clarity, not all concentrations tested are shown. Details of the measurement were as described under Materials and methods. (b) Kinetic plot of the Gls24–CopZ interaction, replotted from the fitted data.

radicals (Kocha *et al.*, 1997). The toxicity of Cu^{+} –CopZ is supported by the findings that CopZ overexpression resulted in increased sensitivity of *E. hirae* to copper and oxidative stress (Lu & Solioz, 2001). One could speculate that Gls24 binds to Cu^{+} –CopZ to protect the exposed copper and/or to present CopZ to a protease for degradation. Such a function of Gls24 would resemble that of SspB of *E. coli*, which is also a partially unstructured, 20-kDa protein induced by nutrient starvation (Levchenko *et al.*, 2000). SspB recognizes SspA-tagged peptides and enhances their degradation by the ClpXP protease system. The partially unfolded structure of Gls24 could conceivably be a key feature for its interaction with CopZ. Clearly, further investigations are required to elucidate the molecular role of Gls24 and other Gls24-like proteins.

Acknowledgements

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Authors' contribution

S.M. and J.V.S contributed equally to this work.

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