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CopY-like copper inducible repressors are putative 'winged helix' proteins

Reto Portmann[1], Kristian R. Poulsen[2], Reinhard Wimmer[3] & Marc Solioz[1,*]
[1]*Department of Clinical Pharmacology, University of Berne, Murtenstrasse 35, CH, 3010, Berne, Switzerland;* [2]*Department of Physics and Nanotechnology, Aalborg University, 9000, Aalborg, Denmark;* [3]*Department of Life Sciences, Aalborg University, 9000, Aalborg, Denmark; *Author for correspondence (Tel: +41-31-632-3268; Fax: +41-31-632-4997; E-mail: marc.solioz@ikp.unibe.ch)*

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Abstract

CopY of *Enterococcus hirae* is a well characterized copper-responsive repressor involved in copper homeostasis. In the absence of copper, it binds to the promoter. In high copper, the CopZ copper chaperone donates copper to CopY, thereby releasing it from the promoter and allowing transcription of the downstream copper homeostatic genes of the *cop* operon. We here show that the CopY-like repressors from *E. hirae*, *Lactococcus lactis*, and *Streptococcus mutans* have similar affinities not only for their native promoters, but also for heterologous *cop* promoters. CopZ of *L. lactis* accelerated the release of CopY from the promoter, suggesting that CopZ of *L. lactis* acts as copper chaperone, similar to CopZ in *E. hirae*. The consensus binding motif of the CopY-like repressors was shown to be TACAxxTGTA. The same binding motif is present in promoters controlled by BlaI of *Bacillus licheniformis*, MecI of *Staphylococcus aureus* and related repressors. BlaI and MecI have known structures and belong to the family of 'winged helix' proteins. In the N- terminal domain, they share significant sequence similarity with CopY of *E. hirae*. Moreover, they bind to the same TACAxxTGTA motif. NMR analysis of the N-terminal DNA binding domain of CopY of *L. lactis* showed that it contained the same α-helical content like the same regions of BlaI and MecI. These findings suggest that the DNA binding domains of CopY-like repressors are also of the 'winged helix' type.

Introduction

With the appearance of oxygen, cells started to make use of copper for functional centers in enzymes to catalyze redox reaction and to fight the toxic effects of oxygen. The usage of copper also has its drawbacks. Elevated intracellular copper levels are toxic for the cell and can cause damage to DNA, proteins and phospholipids. Therefore, cells had to develop mechanisms to tightly regulate copper uptake and export and the intracellular routing of copper. In bacteria, the intracellular copper concentration is controlled by regulation of its uptake and export through the cytoplasmic membrane and by complexation.

Enterococcus hirae, one of the most extensively studied model organism for copper homeostasis, has four copper specific proteins organized in the polycistronic *cop* operon, *copYZAB*. The CopA ATPase can import copper into the cell, the CopB ATPase serves in the extrusion of excessive copper, CopZ is a copper chaperone, and CopY (in the following called ehCopY) a homodimeric, copper inducible repressor. EhCopY regulates the expression of the whole operon (Odermatt *et al.* 1992; Odermatt *et al.* 1994; Odermatt & Solioz 1995; Solioz & Stoyanov 2003). In low copper media, ehCopY is present as a Zn(II) containing homodimer and is bound to the operator/promoter of the *cop* operon (Strausak & Solioz 1997; Portmann *et al.*

2004). There are two binding sites, each one interacting with a ehCopY dimer. These ehCopY binding sites feature the inverted repeat TAC-AnnTGTA, called the 'cop box', which appears to be a conserved motif in copper regulation among *Firmicutes*. When the copper concentration in *E. hirae* is elevated, the copper-loaded chaperone, Cu(I)CopZ, transfers Cu(I) to the CopY repressor, thereby releasing its bound Zn(II). The exchange of one Zn(II) by two Cu(I) per ehCopY monomer abolishes DNA repressor binding, thus allowing transcription of the *cop* operon to proceed (Cobine *et al*. 1999; Cobine *et al*. 2002).

While CopA-, CopB- and CopZ-like proteins have been highly conserved through evolution, organisms evolved different regulatory systems involved in copper homeostasis. For example in *Escherichia coli*, CueR, a transcriptional regulator homologous to MerR, controls the expression of the *E. coli* CopA copper ATPase and the CueO cuprous oxidase (Outten *et al*. 2000; Stoyanov *et al*. 2001; Changela *et al*. 2003). However, in prokaryotes of the phylogenetic group *Bacteria* > *Firmicutes* > *Bacilli* > *Lactobacillales*, copper homeostatic proteins are regulated by copper responsive repressors related to ehCopY. A *cop* operon homologous to that of *E. hirae* was recently characterized in *Streptococcus mutans*, the bacterial etiological agent of dental caries (Vats & Lee 2001). This operon exhibits the same general features of the *E. hirae cop* operon, except for the absence of a CopB-like copper ATPase and a different gene order. *S. mutans* CopY (smCopY) was shown to be a copper-inducible repressor of the operon, which also contains a cop box in the promoter region.

We overexpressed and purified ehCopY, smCopY, and the CopY homologue of *Lactococcus lactis* (llCopY; elsewhere called CopR) and determined the interaction kinetics with their native promoters as well as with homologous promoters by surface plasmon resonance. All pairwise repressor/promoter combinations exhibited kinetic constants within a narrow range, implying high evolutionary conservation of copper regulation by CopY-like proteins. CopZ of *L. lactis* was able to catalyze the release of llCopY from the repressor, supporting a copper chaperone function. Sequence similarity, binding properties, and preliminary NMR analysis suggested that CopY-type repressors belong to the family of winged helix proteins.

Materials and methods

Materials

P-20 (ultrapure Tween-20) was supplied by Biacore and Tris–(2-carboxyethyl)phosphine (TCEP) by Aldrich. All other chemicals were from Sigma or from Merck and were of analytical grade. Restriction enzymes were from Roche. *TaqPlus Long* DNA polymerase was from Stratagene. The following oligonucleotides for binding studies were synthesized by Microsynth (Balgach, Switzerland): biotinylated hirae1 5′-AAGTTAAGTTTACAA-ATGTAATCGATGGAG, complementary hirae2 5′-CTCCATCGATTACATTTGTAAACTTAAC TT, biotinylated control1 5′-TTTTAGTGTTGG-TCCGCAGGAACTTATCTT, complementary control2 5′-AAGATAAGTTCCTGCGGACC-AACACTAAAA, biotinylated mutans1 5′-ATA-ATATATCTACAAATGTAGATGAAAGGA, complementary mutans2 5′-TCCTTTCATCTA-CATTTGTAGATATATTAT, biotinylated lactis1 5′-TTTTAGTGTTTACACGTGTAAACTTATC TT, complementary lactis2 5′-AAGATAAGTTT-ACACGTGTAAACACTAAAA.

Plasmid construction

All amplifications were conducted by PCR with *TaqPlus Long*, used as recommended by the manufacturer. The *copY* gene of *Enterococcus hirae* was amplified from plasmid pWY145 with the primers 5′-GCTTGGATTCTCACCAA-TAAAA and 5′-GGATCCATGGAAGAAA-AGAGAGTATTAATT. The product was cut with *BamHI* and *HindIII* and ligated into pQE8 (Qiagen), digested with the same enzymes, to generate plasmid pWH6, expressing ehCopY with a 6-histidine N-terminal tag.

The gene for llCopY was amplified from genomic DNA of *L. lactis* IL1403 with the primers km3 (5′-GATCGGCGCCATGAACGAAGTAG-AATTTAATG) and km4 (5′-CGATGGATCCTT ATACTCTCCTTTTTAAGCAG). The product was cut with *BamHI* and *NarI*, followed by ligation with pProExHTa (Invitrogen), digested with the same enzymes, to generate plasmid pLlcopy, expressing llCopY with a cleavable 6-histidine N-terminal tag. A plasmid, pLlcopyLT298, expressing only the N-terminal part of llCopY, called llCopYΔC, was constructed by insertion of a stop

codon at position 298 in plasmid pLlcopy with the QuickChange mutagenesis kit (Qiagen).

The gene for smCopY was amplified from genomic DNA of *S. mutans* ATCC700610 using the primers km5 (5′-GATCGGCGCCATGAAAGGAGCTCAAATGACAT) and km6 (5′-CGATGGATCCACAGGTCATGCCATCTATCAAA). The resulting product was cut with *BamHI* and *NarI* and ligated with pProExHTa, cut with the same enzymes, generating plasmid pSmcopy, expressing smCopY with a cleavable 6-histidine N-terminal tag.

The gene for CopZ of *L. lactis* was amplified from genomic DNA of *L. lactis* IL1403 with the primers 5′- CTGTCAGGCGCCATGAAAGAAACTTTAAAAATTG and 5′- GCTCTAGATCAATTCACAAACGAAG. The product was cut with *NarI* and *XbaI* and ligated into pProExHTa, cut with the same enzymes. This resulted in plasmid pZL66, expressing llCopZ with a cleavable 6-histidine N-terminal tag. All plasmids were verified by commercial sequencing (Microsynth).

Protein purification

BL21(DE3) cells (Stratagene) containing plasmid pWH6, pLlcopy, pSmcopy, or pLlcopyLT298 were grown aerobically at 37 °C to an OD_{550} of 0.4. After 4 h of induction with 1 mM isopropyl-β-D-galactopyranoside (IPTG), the cells were collected by centrifugation for 10 min at $5000 \times g$. The cell pellet was washed twice with 200 ml TG buffer (50 mM Tris–SO_4, pH 7.8, 5% (v/v) glycerol) and resuspended in 5 ml of TG buffer/g of wet cells. The cells were broken by 3 passages through a French press at 40 MPa. The cell debris was collected by centrifugation for 1 h at $90\,000 \times g$ and the supernatant applied to a Ni-NTA Superflow (Qiagen) column. CopY was eluted with TG buffer containing 200 mM imidazole. Final purification was achieved by gel filtration on a TSK3000G column in TG buffer. Purity was verified by SDS gel electrophoresis and was greater than 95% for all preparations. The repressor preparations used for DNA interaction studies were shown to be present in the zinc-form by inductively coupled plasma atomic emission analysis.

For NMR analysis, the N-terminal portion of llCopY was expressed from plasmid pLlcopLT298 in *E. coli* BL21(DE3), grown aerobically at 37 °C in minimal media (M9) containing ^{15}N ammonium sulfate (99%, Spectra Stable Isotopes). At an OD_{600} of 0.5, 0.1 mM IPTG was added for induction. After an additional 6 h, cells were harvested by centrifuging 10 min at $6000 \times g$ and resuspended in 50 mM phosphate buffer, pH 8, 300 mM NaCl, and lysed by sonication. Lysates were centrifuged at $25\,000 \times g$ for 1 h and the cleared lysate applied to a Ni-NTA Superflow column. The purified protein was eluded with 50 mM phosphate buffer, pH 8, 300 mM NaCl, 200 mM imidazole. The N-terminal his-tag was removed by treating the protein with AcTEV protease (Invitrogen). The protein was finally purified by gel filtration on a Superdex 75 column (Amersham Bioscience). The protein was concentrated in Centricon tubes.

L. lactis CopZ was overexpressed from pZL66 in *E. coli* BL21(DE3), which was grown aerobically at 37 °C. The cells were induced with 1 mM IPTG for 4 h and the cells were harvested by centrifugation for 10 min at $5000 \times g$. The cells were washed twice with 200 ml of TG buffer, resuspended in 5 ml of TG buffer/g of wet cells, and broken by three passages through a French press at 40 MPa. Cell debris was removed by centrifugation for 1 h at $90\,000 \times g$. The supernatant was applied to a Ni-NTA Superflow column and CopZ was eluted with TG buffer containing 200 mM imidazole. Final purification was achieved by gel filtration on a TSK3000G column in TG buffer.

Removal of the 6-histidine N-terminal tags

The 6-histidine tags of 1 mg of protein in 1 ml were cleaved with 100 U of AcTEV protease (Invitrogen) for 2 h at 30 °C or overnight at 4 °C. Free tags and the AcTEV protease, which was also histidine-tagged, were removed by another passage of the protein through a Ni-NTA Superflow column and collection of the flow-through.

Copper-loading of CopZ

L. lactis CopZ was dialyzed twice against buffer Y (20 mM Tris–acetate, pH 8.0, 5 mM magnesium acetate, 50 mM sodium acetate, 1 mM calcium acetate, 2% acetonitrile, and 0.05% P-20) before being reduced for 15 min at room temperature by adding 1/10 volume of 50 mM TCEP in buffer Y. Cu(I)-acetonitrile, prepared as described

(Hemmerich & Sigwart 1963), was added from a 10 mM stock solution in 2% acetonitrile, 5 mM perchloric acid, to a final concentration of 10 μM. Incubation for 30 min resulted in copper loaded Cu(I)CopZ, which could be stored anaerobically at 4 °C for several weeks.

Surface plasmon resonance analysis

Experiments were performed on a Biacore 1000 instrument, thermostated to 25 °C. Before coupling of DNA to the chip, all four flow cells of a streptavidine coated Biacore SA sensor chip were preconditioned with three consecutive 1-minute injections of 1 M NaCl in 50 mM NaOH, resulting in a baseline of approximately 16 000 response units (RU). Double-stranded oligonucleotides were formed by mixing 500 μl of biotinylated 29-mer oligonucleotide (1.25 μg/ml) with 500 μl of the complementary, non-biotinylated 29-mer oligonucleotide (1.5 μg/ml). The mixture was heated to 80 °C and cooled to 30 °C over an hour in a thermostat. For binding of the biotinylated, double-stranded oligonucleotides to the sensor chip, they were injected at a flow rate of 10 μl/min to obtain an increase of 500 RU above the baseline, which required approximately 5 μl of oligonucleotide solution. Binding experiments were conducted with continuous flow of buffer Y. For regeneration, bound proteins were released from the sensor chip by washing with 10 μl of 2 M $MgCl_2$. Data analysis was performed with the BIAevaluation software version 3.1 (Biacore). All analyzed interactions were assumed to behave according to the Langmuir model ($A + B \leftrightarrow AB$). The calculated association (k_a) and dissociation (k_d) rates were derived by fitting of the biosensor curves. K_D values were calculated from the relation $K_D = k_d/k_a$.

NMR studies

NMR studies were carried out using a Bruker DRX 600 MHz NMR spectrometer with a xyz-grad TXI (H/C/N) probe at 298 K. The HSQC spectrum is recorded with 40 scans and a resolution of 4 K complex data points, with a spectral width of 13 ppm and 512 data points, with a spectral width of 30 ppm in the direct and indirect dimensions, respectively. Spectra were analyzed using xeasy (Bartels *et al.* 1995) and INFIT (Szyperski *et al.* 1992). From the resulting coupling constants, φ angles of the protein backbone can be derived from the relation:

$$J = 6.4 \cdot \cos^2(\varphi - 60) - 1.4 \cdot \cos(\varphi - 60) + 1.9$$

as described (Wüthrich 1986). For the determination of secondary structure from the measured *J*-couplings, the following cut off's were used: < 6, helical structure; > 8.5, sheet structure; 6–8.5, other structures (loops, turns, bends, or random coils).

Results

Surface plasmon resonance analysis of repressor–DNA interaction

Through database searching, organisms containing *cop* operons similar to that of *E. hirae* were identified. In most Gram-positive organisms for which the genome sequence was available, operons containing genes similar to those of the *E. hirae cop* operon could be identified. However, the number and order of these genes differed substantially (Table 1). In *E. hirae*, there are four *cop* genes, arranged in the order *YZAB*. The complete set of these four cop genes could not be detected in any other organism analyzed. *B. subtilis* contains *copZAB* genes, but no *copY* repressor gene, while most other organism only contain a *copZ* chaperone and a *copA* ATPase gene, with or without a *copY*-like repressor gene (for ease of comparison, the gene names were adapted to the *E. hirae* nomenclature). All members of the *Lactobacillales* encoded a copY-like repressor gene, followed by either *copZA* or *copAZ*. Only in *L. sakei*, we could not identify a *copZ* homologue.

Comparison of the CopY-like repressors of *E. hirae*, *L. lactis*, and *S. mutans* revealed extensive sequence similarity between these proteins (Figure 1). They all contain a conserved motif of consensus $CxCx_{4-6}CxC$ in the C-terminal half of the protein. This motif has been shown to be involved in zinc and copper binding in ehCopY. The N-terminal half of CopY-like repressors are presumably involved in DNA binding. We have previously shown that the promoter regions of *Lactobacillus sakei*, *L. lactis*, *E. hirae* and

Table 1. Arrangement of *cop* genes in different Gram-positive bacterial species.

Bacterial Species	*cop* Genes[1]
Bacillus subtilis	ZAB
Clostridium acetobutylium	AZ
Clostridium tentani	AZ
Enterococcus faecalis	YAZ
Enterococcus hirae	YZAB
Fusobacterium nucleatum	ZA
Lactococcus lactis	YZA
Lactobacillus sakei	YA
Methanosarcina mazei	ZA
Oceanobacillus iheyensis	AZ
Staphylococcus aureus / *epidermis*	AZ
Streptococcus agalactiae / *mutans* / *pyogenes*	YAZ

[1]The order of the genes is indicated based on the homologous *E. hirae* genes: A, *copA* copper ATPase gene, B, *copB* copper ATPase gene; Y, *copY* repressor gene; Z, *copZ* copper chaperone gene.

```
CopY E. hirae        1 meekrvlikisdsewevmrviwtlgqanaqqitqiladsm
CopY L. lactis       1 mne--vefnvsnaelivmrviwslgearvdeiyaqipqel
CopY S. mutans       1 mkgaqmt-sisnaewevmrvvwakqmtssseiiailsrty

CopY E. hirae       41 dwkvatvktllgrlvkkealwteqegkkfiyhpavsemen
CopY L. lactis      39 ewslatvktllgrlvkkemlstekegrkfvyrplmeecta
CopY S. mutans      40 cwsastiktlitrlsekgyltsqrqgrkyiyssliseeea

CopY E. hirae       81 vrsatenlfshicakrvgatiadlveeatltqediqqimk
CopY L. lactis      79 inlmadgliqkvcetkhvnvlqemiekstltakdiellqe
CopY S. mutans      80 leqqvsevfsricvtkhqalirhlieetpmtlsdieklea

CopY E. hirae      121 qlnkkep--vetiecncip--gqceckkq-------
CopY L. lactis     119 slnakev--vgqkhcncletsglcackheheqisa-
CopY S. mutans     120 lllskkanavpevkcnci--vgqcsc-yehlevtsk
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Figure 1. Alignment of the CopY homologues used in this study. Regions of identity are highlighted with gray. The cysteine residues of the copper binding motifs are underlined.

S. mutans interact with ehCopY with similar affinities. All these promoters contained the common sequence motif TACAxxTGTA. Mutation of this sequence abolished the CopY–DNA interactions. Thus, this motif appears to be a universal interaction site of CopY-like repressors and was termed 'cop-box'. To test if other CopY-like repressor exhibit similar interaction kinetics, we cloned and purified also the corresponding repressors of *S. mutans* and *L. lactis* and measured the affinities of these repressors for their own and for heterologous promoters by surface plasmon resonance analysis.

Biotinylated repressor DNA probes were coupled to a streptavidin coated Biacore sensor chips to an extent of approximately 500 RU and repressor binding was measured in real-time. Interaction kinetics were assessed by injecting

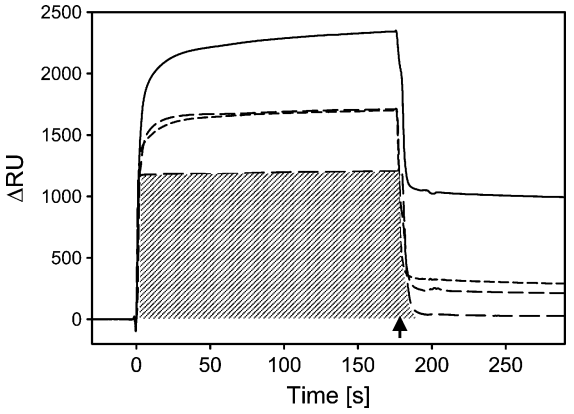

Figure 2. Direct comparison of the interaction kinetics of *E. hirae* CopY with different repressors. At time zero, the injection of 70 μg/ml of CopY into the sensor chip was initiated, followed by buffer only at the *arrow*. The following repressor oligonucleotides were bound to the chip: *E. hirae (—), L. lactis (– – –), S. mutans (- - -), control DNA (– –)*. The shaded area demarcates the jump due do density differences between the buffers.

different repressor concentrations. Figure 2 shows a direct comparison of the interaction of ehCopY with different promoter oligonucleotides as well as with a control oligonucleotide not containing the cop-box. The latter showed no interaction at all with ehCopY. Similar datasets were generated for other repressor–DNA interactions. To obtain reliable kinetic data, association and dissociation rates were assessed at a range of repressor concentrations, as exemplified in Figure 3 for the interaction of llCopY with its native promoter. Table 2 summarizes the kinetic constants derived from the data sets of the repressor–DNA combinations tested. It shows that association and dissociation rates varied in a comparatively narrow range of 0.5×10^4 to 4×10^4 $M^{-1}s^{-1}$ for the k_a values, and of 1.6×10^{-6} to 56×10^{-6} s^{-1} for the k_d values. The resultant K_D values ranged from 1.2 to 40×10^{-10} M.

We have previously shown that in *E. hirae*, Cu(I)CopZ can donate Cu(I) to the repressor, thereby catalyzing its release from the promoter. Since most other Gram-positive organisms also feature genes for CopZ-like copper chaperones in their *cop* operons, it was of interest to see if they can similarly catalyze the release of the repressor from the DNA. To this end, CopZ of *L. lactis* was cloned in *E. coli* and purified to homogeneity. It could be loaded with Cu(I) by the same procedure we had previously used for copper loading of

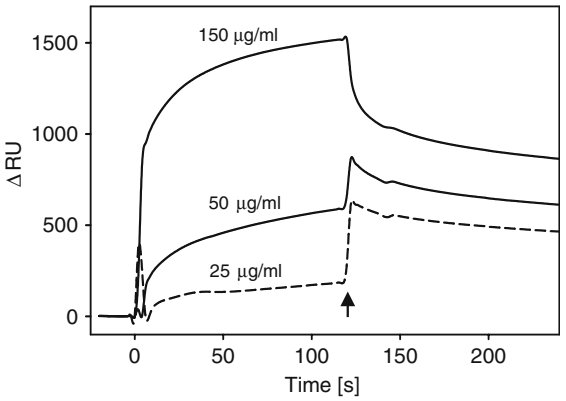

Figure 3. Association and dissociation curves for *L. lactis* CopY–DNA interactions with its native promoter DNA. For each curve, injection of a constant concentration of llCopY into the DNA-coated sensor chip was started at time 0 (association phase), followed injection of buffer only at the *arrow* (dissociation phase). The concentrations of llCopY indicated in the figure were used. This curves were fitted for calculating the affinity constants. Other details were as described under Material and methods.

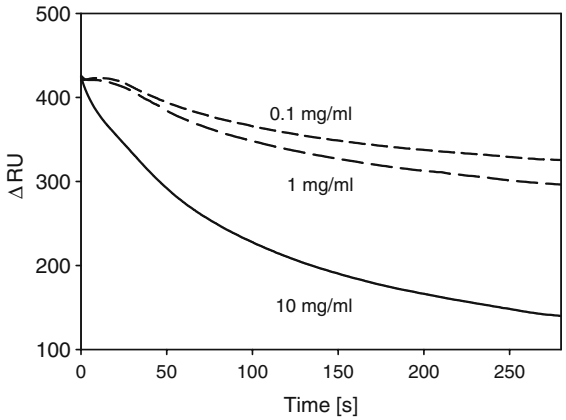

Figure 4. CopZ mediated release of llCopY from the DNA. After injection of *L. lactis* CopY until saturation onto a sensor chip coated with *L. lactis* DNA, *L. lactis* Cu(I)CopZ was injected at the concentrations indicated in the Figure.

E. hirae CopZ (Wimmer *et al*. 1999). Figure 4 shows that *L. lactis* Cu(I)CopZ could catalyze the release of llCopY from its promoter, as has previously been demonstrated by the same technique for the *E. hirae* repressor-chaperone system. It can thus be assumed that CopZ like molecules of other Gram-positive organisms can fulfill the same role as CopZ of *E. hirae*, namely the transfer of Cu(I) to the repressor.

Structure of the DNA binding domain of L. lactis CopY

The N-termini of CopY-like repressors, which presumably form the DNA binding domains, also share sequence similarity with a group of repressors not obviously related to CopY-like proteins, such as BlaI of *Bacillus licheniformis* or MecI of *Staphylococcus aureus* (Figure 5a). BlaI and MecI regulate resistance to β-lactam antibiotics and detailed information on their three-dimensional structure and the DNA–protein interaction is available (Garcia-Castellanos *et al*. 2003; Melckebeke *et al*. 2003; Garcia-Castellanos *et al*. 2004). BlaI and MecI belong to a family of proteins called 'winged helix proteins', based on the structure of the DNA binding domains (Gajiwala & Burley 2000). They consist of three tightly packed α-helices, followed by one or two 'wings' (Figure 5b). The sequence similarity between CopY-like repressors and the BlaI and MecI repressors in the N-terminal domain suggests that CopY-like repressors are winged helix proteins. Interestingly, promoters of the BlaI/MecI family all contain the a consensus DNA motif TAC-AnnTGTA. This motif is identical to the cop-box, further supporting the notion that the DNA binding domains of CopY-like repressors are winged helix proteins.

Table 2. Kinetic constants for CopY–DNA interactions.

CopY Promoter	*E. hirae*[1] *E. hirae*[1]	*L. lactis* *L. lactis*	*S. mutans* *S. mutans*	*E. hirae* *L. lactis*	*E. hirae* *S. mutans*
k_a [$M^{-1}s^{-1}$]	4.3×10^4	0.49×10^4	1.4×10^4	1.4×10^4	1.4×10^4
k_d [s^{-1}]	7.3×10^{-6}	2.5×10^{-6}	1.6×10^{-6}	56×10^{-6}	18×10^{-6}
K_D[2]	1.7×10^{-10}M[1]	5.2×10^{-10}M	1.2×10^{-10}M	40×10^{-10}M	13×10^{-10}M

[1]Data taken from (Portmann *et al*. 2004).
[2]K_D values were calculated according to $K_D = k_d/k_a$.

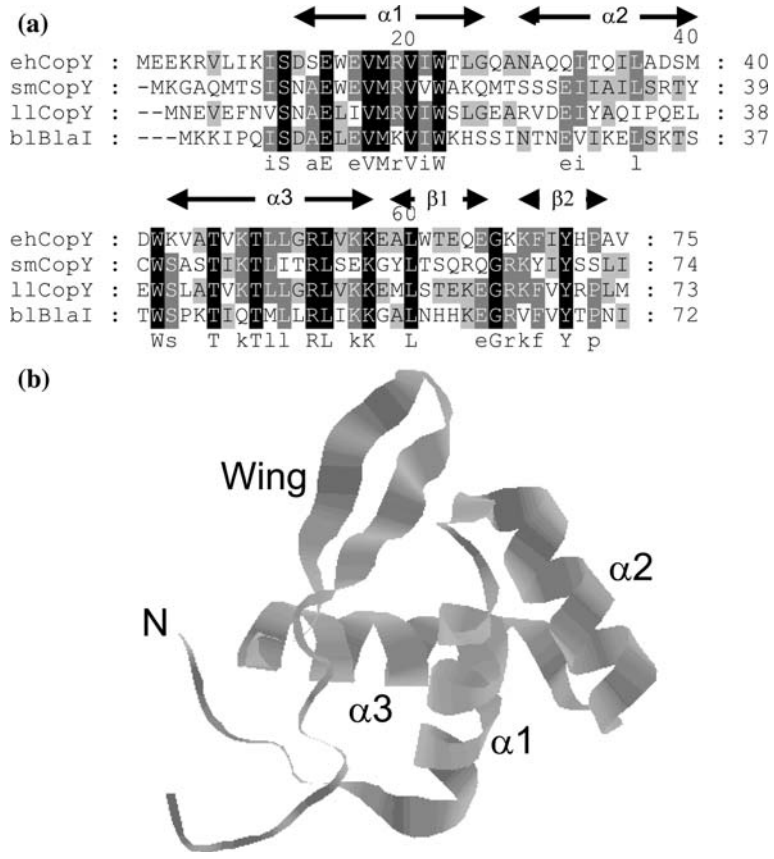

Figure 5. (A). Alignment of putative winged helix DNA binding domains. The DNA binding domains of ehCopY, smCopY, and llCopY were manually aligned with BlaI of B. *licheniformis* (blBlaI). The *arrows* above the sequences indicate the secondary structure elements. Completely conserved amino acids are highlighted with black and partially conserved ones with grey. The weighed consensus sequence is shown below the aligned sequences. (B). Solution structure of the winged helix DNA binding domain (residues 1–82) of BlaI from *L. licheniformis*. The first of the 19 structures contained in entry 1P6R of the PDB protein data bank is shown. N, N-terminus; α1, α2, α3, α-helices 1–3; Wing, wing formed by a hairpin of β-strands β1 and β2.

The CopY-like repressors used in this study as well as that of *Lactococcus sakei* proved to be insufficiently soluble to allow NMR structural work. We therefore constructed a truncated form of llCopZ, called llCopZΔC. This N-terminal fragment comprises the first 74 amino acids of llCopY (77 C-terminal amino acids deleted) plus an extra gly-ala preceding the N-terminal methionine as a result of the his-tag cloning and cleavage strategy. llCopYΔC was still not sufficiently soluble to elucidate its structure by NMR, but it allowed to determine secondary structure elements by *J*-coupling measurements (Figure 6). Table 3 summarizes these data for llCopYΔC, and compares them to the structure content of the same domains of BlaI and MecI. While llCopYΔC exhibits a somewhat lower content of β-sheet structure, the three protein domains exhibit essentially the same α-helical content of 49%. Taken together, these observations suggest that CopY-like repressors are winged helix proteins.

Discussion

The *cop* operon of *E. hirae* is composed of the four genes *copYZAB*, where *Y* encodes the repressor, *Z* the copper chaperone, and *A* and *B* encode copper transporting ATPases. Other Gram-positive bacteria appear to contain similar *cop* operons, featuring two to three of these genes, encoding proteins homologous to the corresponding proteins of *E. hirae*. The CopY repressor of *E. hirae* was previously shown to bind to the consensus

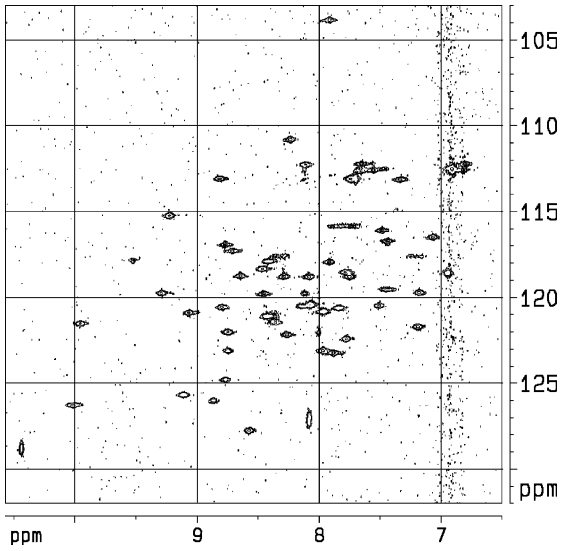

Figure 6. ^{15}N–^{1}H-HSQC NMR spectrum of llCopYΔC recorded at 298 K in 10 mM phosphate buffer, pH 8.

Table 3. Secondary structure content of CopY-like repressors.

Structure	% Contribution		
	llCopYΔC	BlaI[1]	MecI[1]
α -Helix	48.8	49.0	49.6
β -Sheet	9.8	18.7	15.1
Others	41.4	32.3	35.3

[1]Calculated from the structures using MolMol (Koradi *et al.* 1996).

motif TACAnnTGTA (Portmann *et al.* 2004). This motif was also present in the putative *cop* promoter regions of other Gram-positive bacteria. Oligonucleotides corresponding to these promoters were used to measure the binding of the CopY-like repressors from *L. lactis*, *S. mutans*, and *E. hirae* to their own promoters, but also to heterologous promoters. By surface plasmon resonance analysis, it could be shown that the affinities of the different CopY-like repressors for their own promoters fell in the narrow range of 1.2×10^{-10} M to 5.2×10^{-10} M. Affinities for heterologous promoters were lower, but still substantial, with K_D values of 1.3 to 4×10^{-9} M.

Most, but not all *cop* operons in the database feature a CopZ-like putative copper chaperone. In some cases, the CopZ like gene may have been missed because of its small size (200–250 base-pairs), another location, or because the gene does not start with an ATG codon. Parenthetically, a CopZ-like gene has also not been identified in *E. coli*. In *E. hirae*, CopZ delivers copper to eh-CopY to release it from the promoter. It was of interest to see if this was a more general mechanism in Gram-positive bacteria. By surface plasmon resonance analysis, we could show that *L. lactis* Cu(I)CopZ accelerated the release of the *L. lactis* CopY promoter from its DNA target. This strongly suggests that CopZ of *L. lactis* also can donate copper to the CopY repressor for its release from the promoter and it appears likely that this is a general mechanism in Gram-positive bacteria featuring CopY- and CopZ-like genes.

By protein sequence alignment, it became apparent that CopY-like repressors exhibit significant sequence similarity with the repressors Bla1 of *B. licheniformis*, and MecI of *S. aureus*. BlaI and MecI are members of the family of 'winged helix' proteins, which consists of DNA binding proteins with a winged helix structure in the N-terminal DNA binding domain. Winged helix proteins belong to the superfamily of helix-turn-helix DNA binding proteins. Topologically, the winged helix motif is a compact structure, consisting of three α-helices forming the core and two β-strands in a β-hairpin forming the wing (cf. Figure 5). DNA is bound between the helix core and the wing. Other members of the winged helix family feature a second wing (for review, see Gajiwala & Burley 2000). BlaI, MecI, and related regulators of antibiotic resistance, appear to be activated *via* cleavage of the C-terminal dimerization domain, thus preventing dimerization which is required for the interaction with the promoter (Gregory *et al.* 1997; Sharma *et al.* 1998). Other winged helix proteins have different activation mechanisms, such as induction by metal ions. Consequently, the cleavage domain is not conserved in all the winged helix proteins.

For BlaI of *B. licheniformis*, the amino acids affected by DNA binding have been determined by NMR chemical shift comparison between the free and the DNA bound forms. Amino acids in helix 3 were found to make specific contact with the inverted repeat TACAnnTGTA (Melckebeke *et al.* 2003). To the extent that it is known, this DNA motif is conserved in the binding sites of winged helix proteins. We have shown previously that CopY-like repressors feature the identical recognition sequence (Portmann *et al.* 2004). This

further supports the hypothesis that CopY-like repressors are winged helix proteins.

In winged helix proteins of known structure, it could be seen that there are some additional protein–DNA interactions outside of the of the consensus TACAxxTGTA motif. In BlaI of *B. licheniformis*, the motif NHHKE in β-sheet 1 was shown to specifically interact with the AT dinucleotide preceding the TACAnnTGTA motif. The protein sequences in the regions of β -sheet 1 are poorly conserved between different winged helix proteins (cf. Figure 5A). Conceivably, the additional DNA–protein interactions outside of the TACAnnTGTA motif can modulate the affinity of a repressor for its DNA target and may explain the variation in binding affinities of different CopY-like repressors for heterologous promoters.

We attempted NMR structural work on the CopY repressors. However, all the CopYs from different organisms proved to be too insoluble for NMR work. A C-terminally truncated form of llCopY, llCopYΔC was more soluble than the holo protein, but still not at the level required for NMR structural studies. However, we could obtain accurate numbers for the α-helix and β-sheet content for llCopYΔC and compare it to those of other repressors. Interestingly, the N-termini of BlaI, MecI, and llCopY exhibited an essentially identical α-helical content. This furthers supports the notion that llCopY has a structure similar to that of BlaI or MecI and is thus most likely a winged helix protein. Detailed structural work will of course be required for final proof of this hypothesis.

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