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COMMUNICATION

A Conserved Acidic Amino Acid Mediates the Interaction between Modulators and Co-Chaperones in Enterobacteria

Kiran Chintakayala and David C. Grainger*

School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

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Hsp40-like co-chaperones are ubiquitous enzymes that stimulate the protein refolding activity of Hsp70 family chaperones. They are widespread in prokaryotic and eukaryotic systems. In bacteria, the best characterized co-chaperone is the *Escherichia coli* DnaJ protein. Many γ-proteobacteria encode a functional homologue of DnaJ, known as CbpA, which is expressed in response to starvation and environmental stress. The activity of CbpA is regulated by the "modulator" protein CbpM. Here, we have used a combination of genetics and biochemistry to identify the co-chaperone contact determinant of CbpM. We show that the nature of the interaction is conserved in enterobacteria.

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The Hsp70 family of chaperones protects cells against environmental assaults by helping proteins fold accurately. They are found in all bacteria and in all eukaryotic organelles.[1] The activity of Hsp70 proteins can be stimulated by the Hsp40 family of co-chaperones.[2] The best studied chaperone/co-chaperone pairing is the DnaK/DnaJ system of the bacterium *Escherichia coli*.[3] Thus, the DnaJ co-chaperone stimulates the activity of the chaperone DnaK. All DnaJ-like proteins possess an ~70-amino-acid "J-domain" that is essential for co-chaperone activity. In addition to DnaJ, two further J-domain proteins, CbpA and DjlA, can bind DnaK in *E. coli*.[4] It is unclear how association of DnaK with the different J-domain proteins is controlled.

CbpA is a multifunctional protein that was originally identified as a factor present in *E. coli* cell extracts that bound to curved DNA (hence, *c*urved DNA *b*inding *p*rotein *A*).[5,6] It has subsequently been shown that CbpA is also a functional homologue of DnaJ.[6] The CbpA protein is 306 amino acids in length and consists of three domains (Fig. 1a-i). The N-terminal J-domain is required to mediate the interaction with DnaK, the DNA binding activity of CbpA locates to the CTDI domain, and the CTDII domain is required for CbpA dimerization with amino acids W287 and L290 playing a key role[9,11] (Fig. 1a-ii). When over-expressed, CbpA counteracts *dnaJ* phenotypes such as thermal sensitivity and an inability to replicate bacteriophage λ.[6] Consistent with this, there is 39% sequence identity between CbpA and DnaJ with the J-domain of the two proteins being particularly well conserved (55% identical).

The activity of the CbpA is controlled by a low-molecular-weight "modulator" protein called CbpM.[11–14] The *cbpA* and *cbpM* genes form an operon and are co-expressed, and examination of sequenced genomes reveals that this operon is found in a wide variety of bacteria.[12,13] The

*Corresponding author. E-mail address: d.grainger@bham.ac.uk.

Abbreviation used: BACTH, bacterial two-hybrid.

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molecular surface of CbpA that is bound by CbpM locates to the J-domain and primarily consists of positively charged amino acids (R26, R30, and H33)[7,11] (Fig. 1a-ii). This surface overlaps the likely contact determinant for DnaK, suggesting that CbpM modulates CbpA–DnaK interactions and thus indirectly modulates DnaK activity.[7] Despite the similarities between the J-domains of CbpA and DnaJ, CbpM is highly selective and targets only CbpA.[7] We have used a genetic analysis to identify the determinant of the modulator protein CbpM that contacts CbpA. We show that a highly conserved glutamic acid residue, E62, interacts with side-chain H33 in the CbpA J-domain. Furthermore, we show that the nature of co-chaperone–modulator interactions is conserved in enterobacteria.

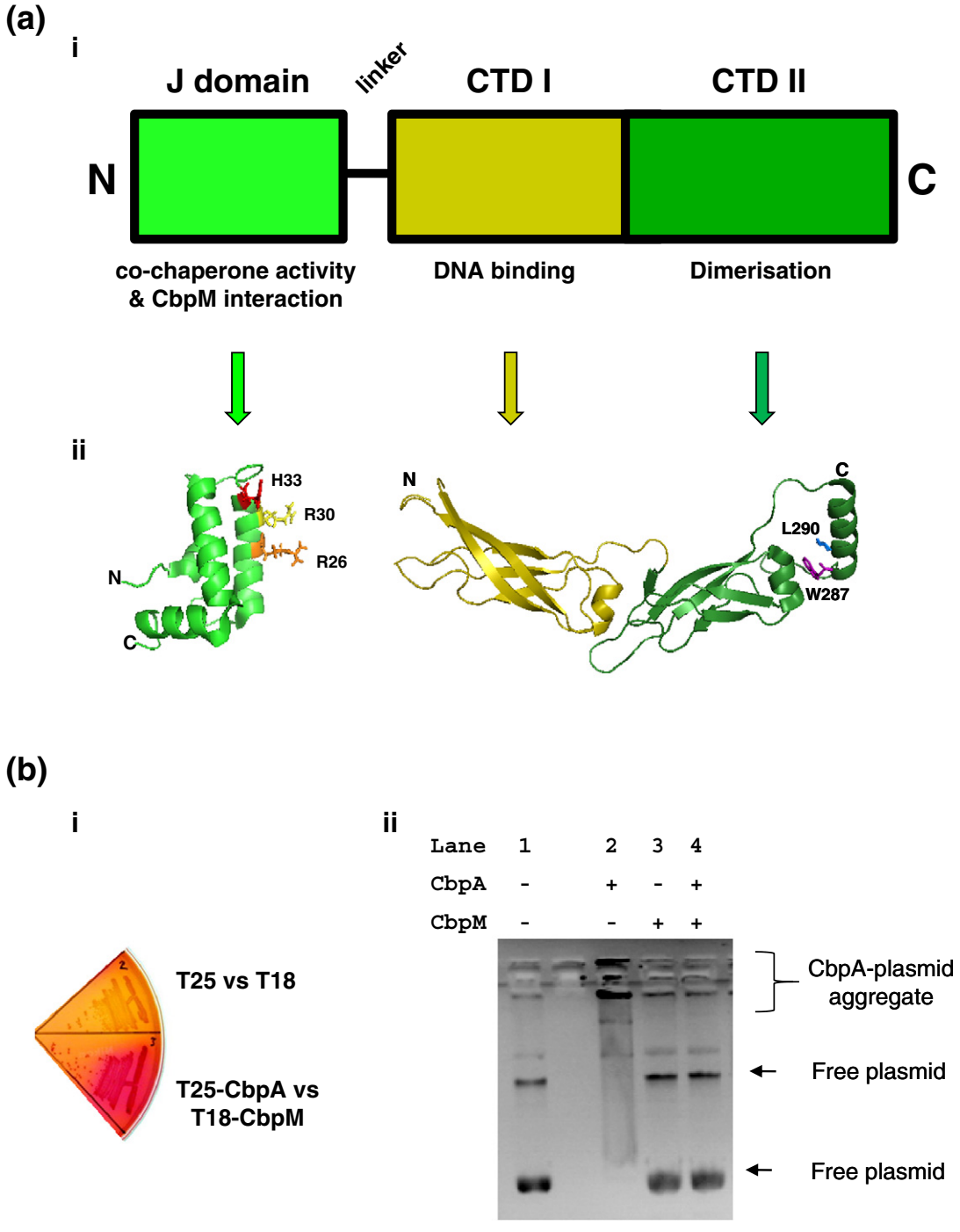

Fig. 1 (*legend on next page*)

In vivo and *in vitro* assays to detect CbpA–CbpM interactions

The starting point for this work was our previous study of dimerization and DNA binding by CbpA.[9] This work revealed that the CbpA–CbpM system was amenable to analysis using the bacterial two-hybrid (BACTH) assay system that detects protein–protein interactions *in vivo*.[15] Note that this system relies on the fact that the *E. coli cyaA*− strain BTH101 is unable to produce cAMP and thus has a Lac− phenotype. This strain can be transformed with plasmids pUT18C and pKT25 that encode two independently folding domains (T18 and T25) of the *Bordetella pertussis* adenylyl cyclase enzyme. When these plasmids are modified, so that T18 and T25 are fused to proteins that interact with each other (in this case, CbpM and CbpA, respectively), a Lac+ phenotype is conferred upon the cell (Fig. 1b-i). Interactions can be quantified *in vivo* by measuring β-galactosidase activity. Our previous work also demonstrated that CbpA forms aggregates when bound to DNA that can be detected *in vitro* using gel shift assays.[9] Figure 1b-ii shows the outcome of such an experiment. Addition of CbpA to plasmid DNA results in the formation of aggregates that are trapped in the wells of the gel (compare lanes 1 and 2). Conversely, CbpM is unable to bind DNA (compare lanes 1 and 3) but is able to disrupt CbpA–DNA aggregates (compare lanes 2 and 4). Thus, this "disaggregation" assay can be used to monitor CbpA–CbpM interactions *in vitro*.

Charge reversal substitutions that abolish CbpA–CbpM interactions

Sarraf *et al.* used NMR spectroscopy and surface plasmon resonance to identify the region of CbpA that is perturbed upon interaction with the modulator protein CbpM.[7] The surface locates to the J-domain and includes positively charged amino acid side chains R26, R30, and H33 (Fig. 1a-ii). To test the observations of Sarraf *et al.*, we utilized the BACTH assay.[7] Thus, the plasmid encoding the T25-CbpA fusion was modified to encode T25-CbpA with charge reversal substitutions (R26E, R30E, or H33D) in the CbpA J-domain. We then measured the ability of the different T25-CbpA derivatives to interact with T18-CbpM. The results show that the H33D and R26E substitutions abolish the CbpA–CbpM interaction, while the R30E substitution reduced the interaction by threefold (Fig. 2a-i). Since CbpA dimerization does not involve the J-domain, we reasoned that CbpA dimerization should not be affected by mutations in the CbpM contact surface. Thus, we also used the BACTH system to measure interactions between the different T25-CbpA derivatives and a wild-type T18-CbpA fusion. The results unexpectedly show that the R26E and R30E substitutions reduced CbpA dimerization by twofold (Fig. 2a-ii). However, as expected, the H33D substitution did not disrupt CbpA dimerization (Fig. 2a-ii). We reasoned that the co-chaperone contact determinant in CbpM would most likely contain negatively charged amino acids. Thus, the 15 negatively charged side chains in *E. coli* K-12 CbpM were sequentially replaced with positively charged amino acids. We then measured the effects of these substitutions on the CbpM–CbpA interaction using the BACTH system. The data show that five substitutions in CbpM (E23K, D46K, D47K, E62K, and D66K) abolished the CbpA–CbpM interaction (Fig. 2b).

Identification of mutations in CbpM that suppress the effect of CbpA^{H33D}

We next sought to pinpoint the co-chaperone contact determinant in CbpM. Thus, we sequentially combined all of the various charge reversal

Fig. 1. Domain organization of CbpA and detection of interactions with its modulator CbpM. (a) Domain organization and interaction determinants of CbpA. (a-i) Individual domains of CbpA are shown as different colored boxes, and the linker between the J-domain and CTDI is shown as a line. The function of each domain is indicted below the diagram. (a-ii) The panel shows structures for the *E. coli* K-12 CbpA J-domain[7] and the *Klebsiella pneumoniae* CbpA CTDI-CTDII fragment (84% identical with the *E. coli* protein).[8] The domains are colored according to the scheme in (a-i). The amino acid side chains required for dimerization (W287 and L290) and the interaction with CbpM (R26, R30, and H33) are highlighted. (b) Detection of CbpA–CbpM interactions. (b-i) A BACTH system can be used to detect CbpA–CbpM interactions. The panel shows a photograph of BTH101 cells, carrying different derivatives of the pKT25 and pUT18C plasmids, growing on a MacConkey agar indicator plate. (b-ii) A disaggregation assay can be used to monitor CbpA–CbpM interactions *in vitro*. CbpA protein (at a concentration of 2 μM, purified as we described previously[9]) was incubated with 150 ng of pSR plasmid DNA in 20 mM Tris (pH 7.0), 10 mM $MgCl_2$, 100 μM ethylenediaminetetraacetic acid, and 120 mM KCl for 10 min at 25 °C before being mixed with gel loading dye and loaded onto a 1% (w/v) agarose gel containing 1 μg/ml ethidium bromide. Gels were run for 45 min at 80 V. Where present, CbpM was added at a concentration of 2 μM to either naked DNA or preformed CbpA–DNA aggregates. To purify CbpM, we cloned the *cbpM* gene from *E. coli* K-12 into vector pET21a and over-expressed it in T7 express cells (Invitrogen). Cells were harvested by centrifugation, resuspended in buffer FB,[10] and then lysed by sonication. The CbpM protein formed inclusion bodies that were resolubilized in 4 M urea. Urea was then removed by dialysis, and precipitated protein was removed by centrifugation. Refolded protein was purified using a gel-filtration column. Primer sequences for cloning of *cbpM* and derivatives are available on request.

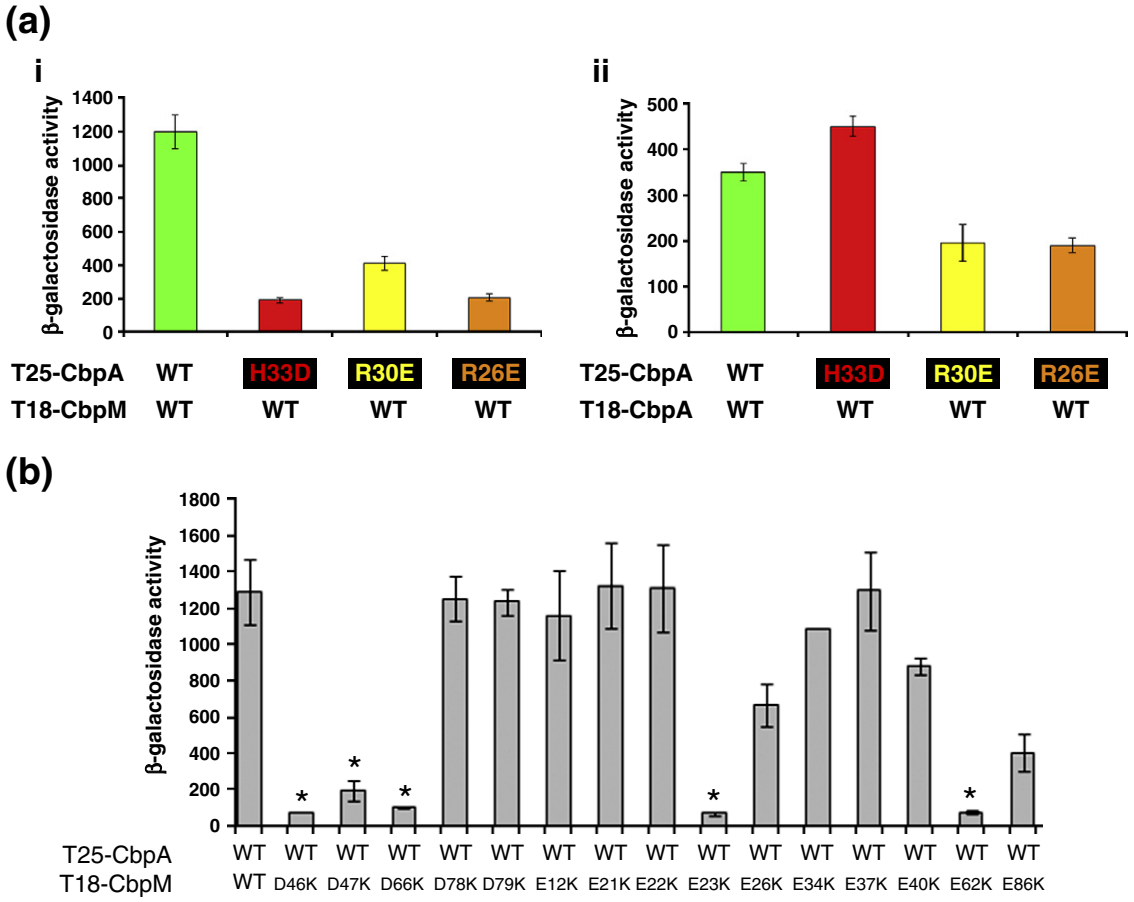

Fig. 2. Effect of charge reversal substitutions on CbpA–CbpM interactions. (a) Charge reversal substitutions in CbpA. (a-i) The bar chart shows results of BACTH assays performed to measure CbpA–CbpM interactions. We determined β-galactosidase levels in overnight cultures of BTH101, carrying derivatives of pKT25-CbpA and pUT18C-CbpM, by the Miller method.[16] Activities are shown in Miller units and are the average of three or more independent experiments. All assays were performed using cells grown in MacConkey broth as described previously.[9] The different CbpA derivatives tested are indicted below the chart. (a-ii) The graph shows results of BACTH assays performed to measure CbpA–CbpA interactions. We determined β-galactosidase levels in overnight cultures of BTH101 carrying derivatives of pKT25-CbpA and pUT18-CbpA as described above. The different CbpA derivatives tested are indicted below the chart. (b) Charge reversal substitutions in CbpM. The graph shows results of BACTH assays performed to measure CbpA–CbpM interactions. We determined β-galactosidase levels in overnight cultures of BTH101 carrying pKT25-CbpA and derivatives of pUT18C-CbpM. The different CbpM derivatives tested are indicated below the chart. The stars highlight the CbpM mutations studied further in this work.

substitutions in CbpM with those in CbpA. Our logic was that reversing the charge of one contact surface would abolish the interaction but reversing the charge of both surfaces would not. As expected, the vast majority of combinations resulted in no detectable CbpA–CbpM interaction (Fig. 3a). Strikingly, when the CbpA^{H33D} and CbpM^{E62K} substitutions were combined, the interaction was restored (Fig. 3a). Thus, CbpA^{H33D} and CbpM^{E62K} can interact with each other but not with the wild-type versions of their cognate interaction partner. The CbpA^{H33D} and CbpM^{E62K} derivatives were over-expressed, purified, and analyzed *in vitro* using the disaggregation assay described above in order to confirm that this observation was not a quirk of the two-hybrid analysis. The results of the analysis show that CbpA^{H33D} forms aggregates with DNA (Fig. 3b, lanes 1 and 2) but that wild-type CbpM cannot effectively disrupt the aggregates (lane 3). Conversely, the aggregates are efficiently disrupted by CbpM^{E62K} (lane 4). We also found that CbpM^{E62K} was unable to interact with CbpA in cross-linking experiments *in vitro* (Supplementary Fig. 1).

The CbpA–CbpM contact is evolutionarily conserved

Using the BACTH system, we examined the interaction between the CbpA and CbpM proteins encoded by *Shigella flexneri* or *Yersinia enterocolitica*.

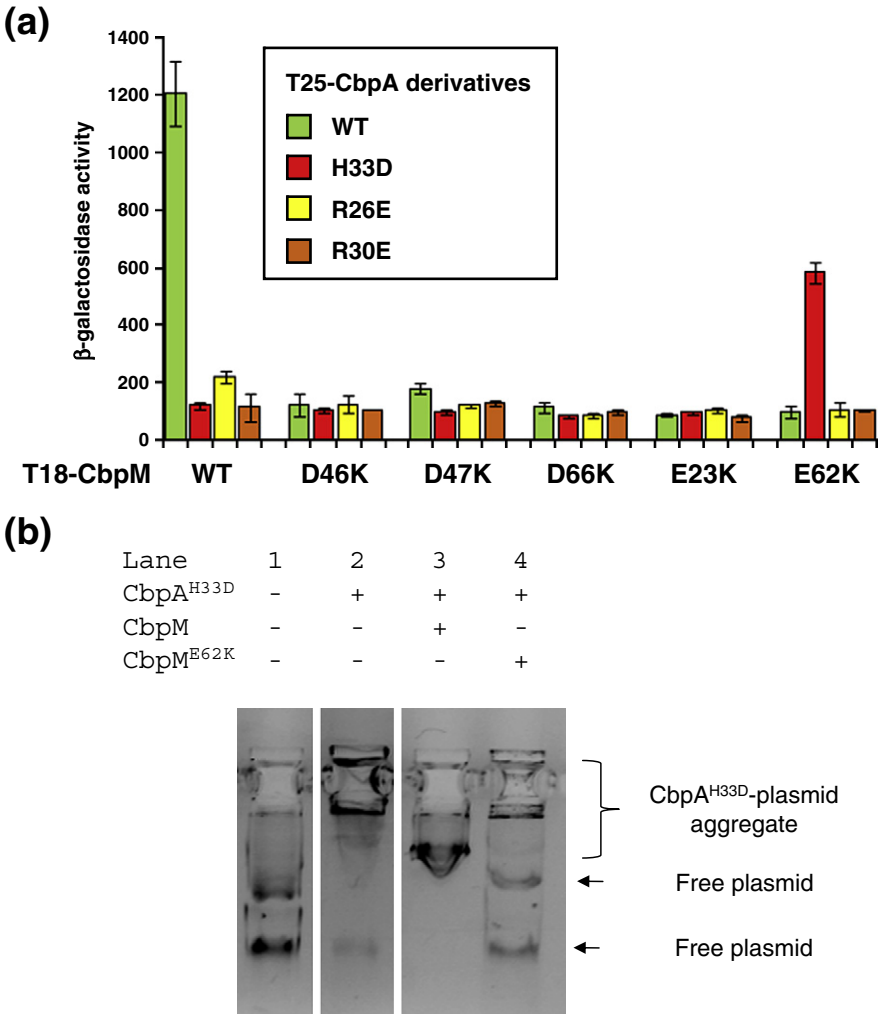

Fig. 3. CbpM side-chain E62 mediates the interaction with CbpA residue H33. (a) Combining different charge reversal substitutions in CbpA and CbpM. The bar chart illustrates results of BACTH assays performed to measure CbpA–CbpM interactions. We determined β-galactosidase levels in overnight cultures of BTH101 carrying different combinations of pKT25-CbpA derivatives and pUT18C-CbpM derivatives. The different CbpM derivatives tested are indicated below the chart. The color of individual bars on the chart varies according to the CbpA derivative tested (see color key). (b) $CbpA^{H33D}$ and $CbpM^{E62K}$ interact *in vitro*. The panel shows a photograph of an agarose gel on which different plasmid DNA–protein complexes have been analyzed. $CbpA^{H33D}$ and $CbpM^{E62K}$ were purified as described above.

For both organisms, the interaction required CbpA side-chain H33 and CbpM side-chain E62 (Supplementary Fig. 2). Thus, we reasoned that the nature of the CbpA–CbpM interaction must be evolutionarily conserved. Figure 4a shows a phylogenetic tree for organisms with a *cbpAM* operon. We expected that, if the nature of the modulator–co-chaperone interaction was highly conserved, modulator proteins from other bacteria should be able to interact with *E. coli* K-12 CbpA. Moreover, the interaction should remain dependent on side-chain E62 (or equivalent) in the various CbpM proteins. We selected CbpM homologues from four enterobacteria (*Shigella dysenteriae*, *Citrobacter koseri*, *Salmonella typhimurium*, and *Y. enterocolitica*) and one from a more distantly related organism (*Pseudomonas putida*) for our analysis (see organisms highlighted by arrows in Fig. 4a). Note that the CbpM homologues from enterobacteria are 99%, 77%, 75%, and 53% identical with the *E. coli* K-12 CbpM protein, respectively. The *P. putida* CbpM protein is 38% identical with *E. coli* K-12 CbpM. We tested the ability of these different CbpM homologues to interact with *E. coli* K-12 CbpA using the BACTH system. The data (Fig. 4b) show that all of the CbpM homologues from enterobacteria were able to interact with the *E. coli* K-12 CbpA protein, and in all cases, the interaction was abolished by the E62K mutation in CbpM. The *P. putida* CbpM protein did not interact with *E. coli* K-12 CbpA. However, on further investigation, we

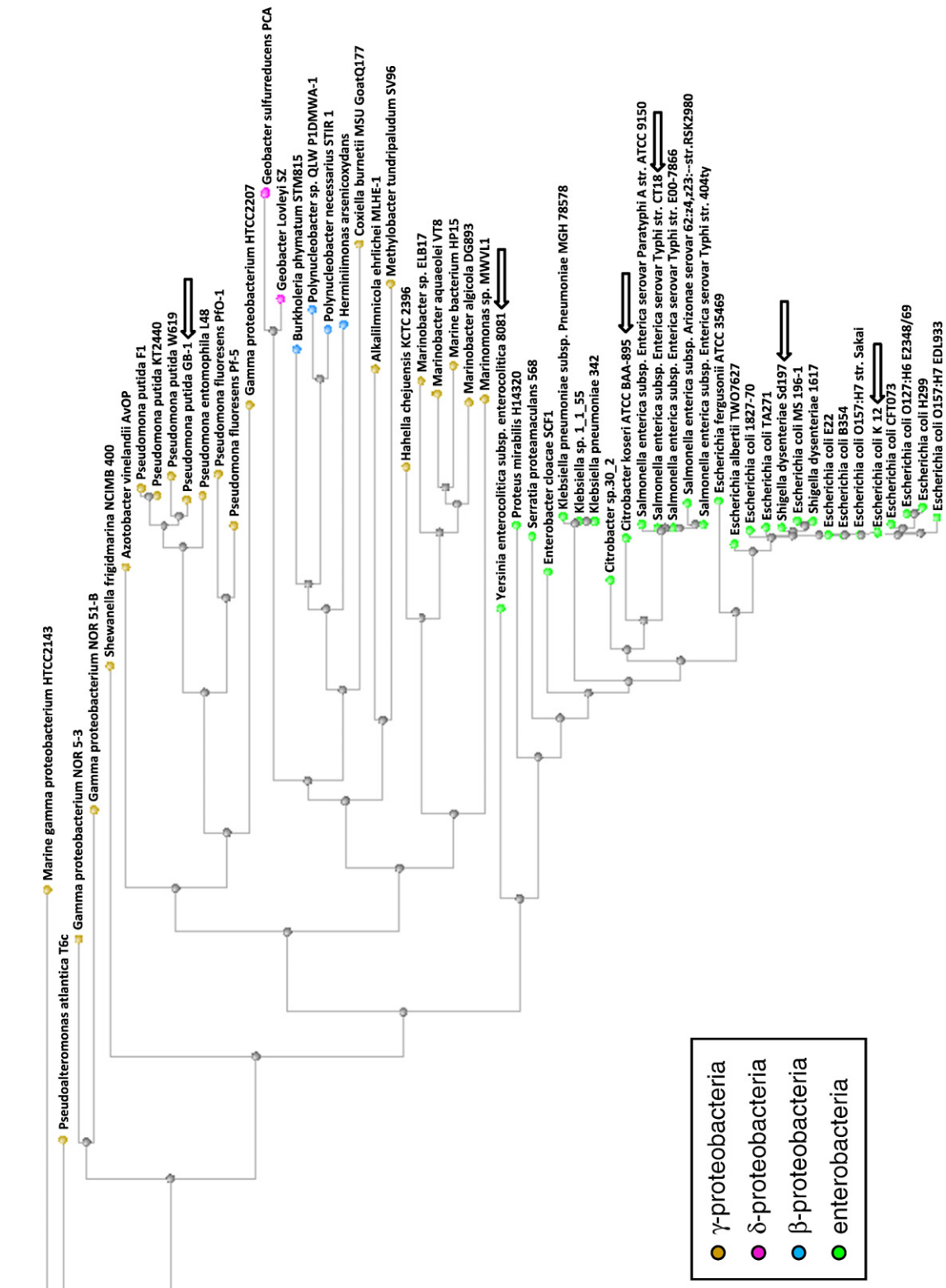

Fig. 4 (*legend on next page*)

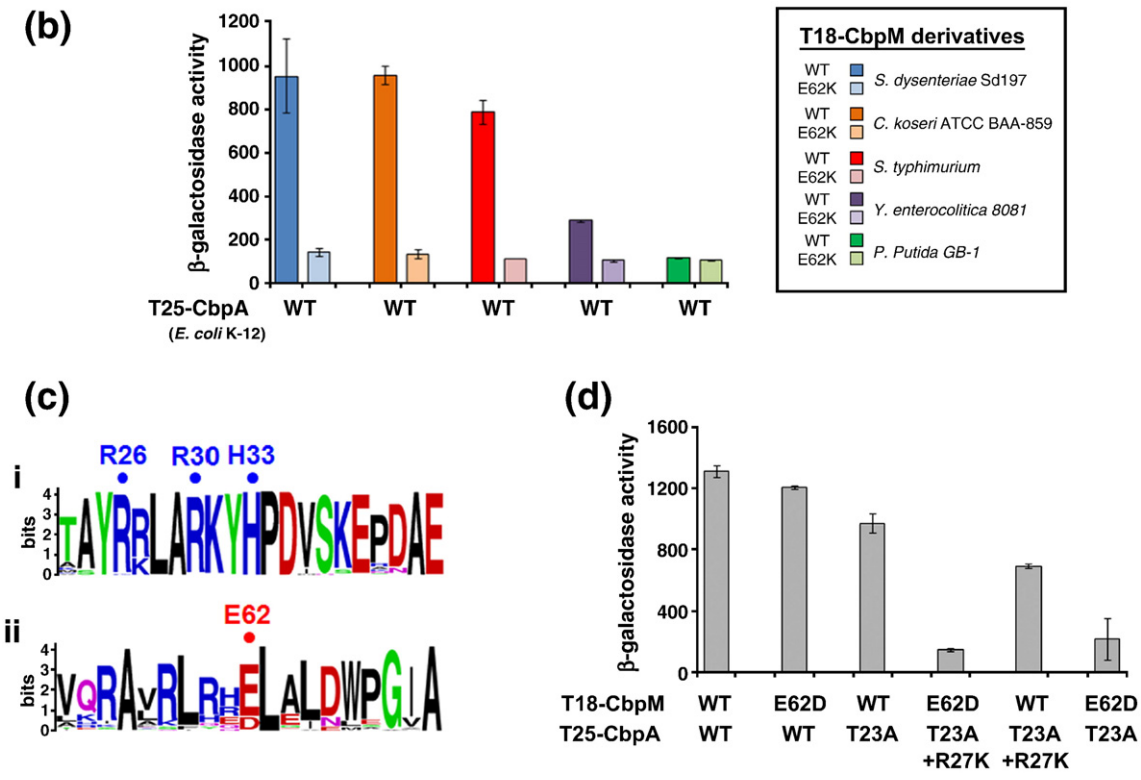

Fig. 4. Cross-species CbpA–CbpM interactions. (a) Phylogenetic analysis of organisms containing a *cbpAM* operon. The panel shows a phylogenetic tree for a range of organisms containing a copy of the *cbpAM* operon. For simplicity, we have not shown very closely related organisms (e.g., different *E. coli* K-12 strains). Organisms from which CbpM homologues were studied further are highlighted by arrows. (b) Interspecies CbpA–CbpM interactions require CbpM side-chain E62. The bar chart illustrates results of BACTH assays performed to measure interactions between the *E. coli* K-12 CbpA protein and CbpM proteins from different bacteria. The β-galactosidase levels were determined in overnight cultures of BTH101 carrying combinations of pKT25-CbpA and different pUT18C-CbpM derivatives. The different CbpM derivatives tested are indicated in the color key shown alongside the chart. The genes encoding the different CbpM homologous and their derivatives with an E62K mutation were purchased from DNA2.0 (California, USA) and were cloned into plasmid pUT18C as described previously.[9] (c) Alignment of the CbpA and CbpM interaction surfaces from different bacteria. The panel shows protein sequence logos generated by aligning (c-i) CbpA homologues or (c-ii) CbpM homologous from different bacteria. The sequence logos are centered around the proposed CbpA–CbpM interaction surfaces. (d) Conservative changes in the CbpA–CbpM interaction surface are tolerated in only one interaction partner. The panel shows results of BACTH assays performed to measure interactions between derivatives of the *E. coli* K-12 CbpA and CbpM proteins. The different CbpA and CbpM derivatives tested are indicated below the chart.

found that the *P. putida* CbpM also failed to interact with its cognate CbpA (data not shown). As expected, *E. coli* K-12 CbpA^{H33D} could not interact with any of the different CbpM proteins (Supplementary Fig. 3).

Comparison of CbpA and CbpM proteins from different bacteria reveals that minor variations in the amino acid sequence can occur close to the interaction determinants of the two factors. This is illustrated in Fig. 4c, which shows sequence logos generated by aligning CbpA (Fig. 4c-i) and CbpM (Fig. 4c-ii) homologues from different bacteria. For example, in some CbpM proteins, the crucial E62 side chain is replaced with D. Similarly, some CbpA proteins exhibit changes in their sequence adjacent to the key amino acids R26, R30, and H33. Thus, in CbpA, T23 is replaced with A, and R27 is replaced with K in some instances. Guided by the protein sequence logos in Fig. 4c, we made conservative changes close to the *E. coli* K-12 CbpA and CbpM interaction surfaces. We found that when changes were made only in CbpA or only in CbpM, there was little or no effect. Strikingly, when the changes were made in both CbpA and CbpM, the interaction was completely abolished (Fig. 4d).

Conclusions

The *cbpAM* operon is widely conserved in γ-proteobacteria, particularly among pathogenic

enterobacteria.[12] The ability of CbpM to modulate the activity of CbpA was first document by Chae *et al.*,[12] and it has subsequently been shown that CbpM represses both the DNA binding and co-chaperone activities of CbpA[11,12] (Fig. 1b-ii). A H33D charge reversal substitution in the CbpA J-domain completely abolishes the ability of CbpA to interact with CbpM (Fig. 2a-i) but has no effect on CbpA dimerization (Fig. 2a-ii). Hence, CbpA residue H33 must make a crucial contact with CbpM. The E62K substitution in CbpM suppresses the effects of CbpA H33D mutation (Fig. 3) but abolishes the interaction of CbpM with wild-type CbpA (Fig. 2b). We conclude that CbpA side-chain H33 and CbpM side-chain E62 directly interact. We note that the R26E and R30E mutations in CbpA, previously shown to locate to the CbpA–CbpM interface, disrupt both the interaction with CbpM and (to a lesser extent) CbpA dimerization. This is surprising since the CbpA dimerization and CbpM interaction determinants are located at opposite ends of the CbpA molecule (Fig. 1a). We conclude that the R26E and R30E mutations induce changes in CbpA conformation. This may explain our inability to identify mutations in CbpM that suppressed the effects of the R26E and R30E substitutions in CbpA (Fig. 3a).

A secondary aim of this work was to determine whether the nature of the CbpA–CbpM interaction was conserved in enteric bacteria. Our data show that *E. coli* K-12 CbpA is able to interact with CbpM homologues from even the most distantly related enteric bacteria, suggesting a conserved mechanism of interaction (Fig. 4a and b). Moreover, the $CbpM^{E62K}$ substitution abolished the interaction in all cases (Fig. 4b). We note that even subtle changes in the CbpA–CbpM interaction surface are deleterious when made in both of the interacting proteins (Fig. 4d). This likely explains why *E. coli* CbpA interacts less efficiently with CbpM proteins from more distantly related organisms (Fig. 4b). In summary, CbpM side-chain E62 interacts directly with CbpA side-chain H33, and the mechanism of CbpA–CbpM interaction is conserved in enteric bacteria.

Supplementary materials related to this article can be found online at doi:10.1016/j.jmb.2011.05.043

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