Independent Evolution of Heavy Metal-Associated Domains in Copper Chaperones and Copper-Transporting ATPases

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Abstract. Copper chaperones are small cytoplasmic proteins that bind intracellular copper (Cu) and deliver it to Cu-dependent enzymes such as cytochrome oxidase, superoxide dismutase, and amine oxidase. Copper chaperones are similar in sequence and structure to the Cu-binding heavy metal-associated (HMA) domains of Cu-transporting ATPases (Cu-ATPases), and the genes for copper chaperones and Cu-ATPases are often located in the same operon. Phylogenetic analysis shows that Cu chaperones and HMA domains of Cu-ATPases represent ancient and distinct lineages that have evolved largely independently since their initial separation. Copper chaperone–Cu-ATPase operons appear to have evolved independently in different prokaryotic lineages, probably due to a strong selective pressure for coexpression of these genes.

Key words: P-type ATPases — Cation transport — Metalloenzyme — Copper poisoning — Wilson disease — Menkes disease — Mercury-binding protein

Introduction

Copper (Cu), like other transitional metals, is involved in a number of important redox reactions in the cellular energy metabolism. Cu-dependent enzymes, such as cytochrome oxidase (EC 1.9.3.1), superoxide dismutase (EC 1.15.1.1), and amine oxidase (EC 1.4.3.6), are found in many organisms (McGuirl and Dooley 1999). However, Cu ions are toxic, and their cellular concentration has to be kept very low (Dameron and Harrison 1998; Rae et al. 1999). The task of sequestering intracellular copper and delivering it specifically to the Cu-dependent enzymes is accomplished by Cu chaperones, small cytoplasmic proteins that bind Cu ions with a $K_d < 10^{-20}$ M (Rae et al. 1999). Cu chaperones are similar in sequence and structure to the Cu-binding heavy metal-associated (HMA) domains of Cu-transporting ATPases (Cu-ATPases) whose genes are mutated in human Wilson and Menkes diseases (Bull et al. 1993; Solioz et al. 1994; Vulpe et al. 1993). Cu chaperones have been characterized in yeast, worm, and humans (Culotta et al. 1997; Harrison et al. 1999; Hung et al. 1998; Pufahl et al. 1997; Wakabayashi et al. 1998), where their activity has been studied as part of the efforts to better understand Cu metabolism aberrations in familial amyotrophic lateral sclerosis (Corson et al. 1998; Culotta et al. 1997). In bacteria, a Cu chaperone has been characterized in *Enterococcus hirae* (Cobine et al. 1999; Odermatt and Solioz 1995), and related mercury-binding proteins (HgBPs) have been identified in the periplasm of several bacteria harboring mercury-resistance plasmids (Bhriain and Foster 1986; Silver et al. 1989). A recent survey of the distribution of Cu chaperones in completely sequenced genomes showed that they are encoded by most free-living bacteria and some archaea (Jordan et al. 2000). The genes for Cu chaperones are usually clustered and presumably form operons with the genes that encode Cu-ATPases containing HMA domains at their N termini (Fagan and Saier, 1994; Silver, 1996; Rensing et al.

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Copper chaperones and corresponding ATPases

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YvgY_Bs    (69)    1 MEQKTIQVEGMSCQHCVKAVETSVGELDGVSAVHVNLEAGKVDVSFDADKVSVKDLADAIEDQGYDVAK    69 2635864
YvgX_Bs   (803)    5 QKEIAMCVSGMTCAACANRIEKGLKRMPGVTDANVNLATETSNVIYDPAETGTAALQEKIEKLGYHVVT    73 2635863
                  74 -EKAEDIEGHTCAACANRIEKFLNKIEGVANAPVNFALETVTVEYNPKEASVSDLKEAVDKLGYKLKL   131 2635863
YvgW_Bs   (702)    3 LVKQEVTVDGLDCSNCARKIENGVKGIKGINGCAVNFAASTLTVSADGKEEQMVTNKVEKKVKSIDPHV    71 2635862

Cj1162c    (64)    1 ---MKFKVENMNCNHCVNLIKMSLEDEFGNIEVDLEQKILSLNLEENQ----VSNFTKFQDLGFEIVE    62 6968595
Cj1161c   (699)    1 MEELRIKIGHMTCVNCSNAIERACKKIDGVKDASVSVVNSSGVFLLED-QKKRKDIIAKIKNLGFEILE    68 6968594
Cj1155c   (785)   15 AQMIEYKGKFFCGKGAESVVEILHESGLDEFYEKLGNQTLSPVNFQNE--MKNYDEPITKTKEGFSEIY    81 6968588
                  82 -----LMIHGIECAACVWLNEKILTKQEGILELDINHLNHKARIVFDEQSISLVQLRLIESIGYKASA   143 6968588

DR2452     (68)    1 -MQTELNVTGMSCGHCVKAVEGALKAVPGVEGVQWSLBGGKATVQGDA----DAQALIAAVKEEGYGAEV    65 6460267
DR2453    (843)    1 MKTLDLDISGMTCAACVGRVERGLRKVEGVQEAHVNLATERASVTYDPALTNAAALVQKVVDTGVDAPT    69 6460273
                  70 -AELSFPVAGMTCAACVGRVERALNKTDGVLDASVNLATERASVRYLPASVSPAELKSAVVNAGYDVPE   137 6460273

HI0292     (68)    1 MKTITLNIKGIHCGCVKSLTQVLTELDGVQSADVQLE-GKANITFDENRVNVAQLIEVIEDAGFDATE    68 1573260
HI0291     (68)    1 MKTITLNIKGIHCGCVKNLTQVLTELDGVQSADVQLE-GKANITFDENRVNVAQLIEVIEDAGFDATE    68 1573259
HI0290    (722)    9 SKKISIQIGGMTCQSCANRIEKVLNKKPFVQQAGVNFAAEEAQVVFDATQASEAQIIEIHKTGFSAHI    74 2493017
HI1050    (92)    23 TKQIVLKVKEMCQLAAYLVNKELRNINGVISTKASIKDGLVTVVEDP-NVTNQQLFDAIHKLKYTAEV    90 1574083

HP1073     (66)    1 -MKATFQWPSITCNECVDKIEKFVGEIEGVSFIDVSVEKKSVVVFBDA-PATQDLIKEALLDAGQEVV-    66 3121871
HP1072    (745)    1 -MKESFYIEGMTCTACSSGIERSLGRKSFVKKIEVSLLNKSAMIEFDENQTNLDEIFKLIEKLGTSPKK    68 2314221

NMB1271    (70)    1 METLIIDIGGMSCGGCVKSVTRILEGVKGVASVETSLENKSATVGYDPAQTDAGALIEAVEDGGYDAAL    69 7226510
NMB1325   (720)    2 QQKIRFQIEGMTCQACASRIEKVLNKKDFVESAGVNFASEEAQVVFDDSKTSVADIAKIIEKTGYGAKE    70 7226567
NMB1042   (823)   19 TVRIENEDRETCQAGCQAVAQSIIDAGLGSYYKQRTADAQRTELPPQEILDQIRLVDLPEVQSDFVETH    87 7226281
                  90 TREAVLMLGGITCAACVWLIBQQLLRTDGIVRIDLNVSTHRCRVMDDGKIRLSDILLKIRQIGYTAAP   158 7226281

TM0320     (67)    1 -MRIVLYVPDISCNHCKMRISKALEEL-GVKNYEVSVEEKKVWVETEN----LDSVLKKLBEIDYPVES    63 4980821
TM0317    (726)   16 EIKKTFTVTGMZATCAKTVEKALKKLDGVKFAAVNLATSTGFIVAEK-EISPEEIKKAVEEVGTGVTT    83 4980818

Ssr2857_Sy (64)    1 -MTIQETVNPTIACEACAEAVTKAVQNEDAQATVQDLTSKKVTITSAL---GEEQLRTAIASAGHEVE-    64 1652317
Sll1920_Sy(745)    3 QTILQLEGMECAACASSIERAIAKVPGVQSCQVNFALEQAVVSYHG-ETTPQILTDAVERAGYHARV    69 2493001

APE0009   (105)   29 QEKALLGIEGMECEGCAIAIBTALKNVKGIIDTKVNISRGSAIVTFDDTLVSINDILEHYIFKVPSNYR    96 5103397
APE1454   (835)    3 LVKEKIIKGMPGATCGLTVEKALRSLPGVVEASADPVTGEAVIATEPGSVRLSDVVKAIRSVGYDVVL    71 5105138

Aq_1840a   (68)    1 MGEKEKIQGMTCQHCVNTVKRALSEVEGVSEVEVSIEBGKAKVXLEK-EWPFETLKEAVETWGYKVVE    68 -------
Aq_1445   (679)    1 -MRKAFKVYGMSCVNCARAIEITLKRTBGVKNVNVSFELGRVEVEFDEDLISEEEIVKKIEBLGYTSVER    68 2983833

UU202      (74)    1 MTTVMLDTSSDLPGGSCSSNLYNVLEKI-GAQNISVNILNSEFAFEFEENKIADQDVIKEINKNGFKTNI    70 6899168
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Fig. 1. Alignment of heavy metal-associated domains encoded in the complete genomes. The proteins are listed under their names in complete genomes (*left column*) and their unique identifiers in the NCBI protein database (*right column*); the total sizes of the proteins are given in parentheses. The numbers at the left and right indicate the positions of the first and the last amino acid residues in the alignment. The names of the HMA domains from experimentally characterized proteins or with known 3D structures are in *boldface*. Conserved amino acid residues are shown in *boldface*. *White letters on a black background* indicate conserved Cys residues that are involved in metal binding. *Light gray shading* indicates conserved hydrophobic amino acid residues. The structural assignments are from the crystal structures of the HMA domains of the human Menkes protein [PDB code 1AW0 (Gitschier et al. 1998)]; yeast Cu chaperones ATX1 [PDB code 1CC7 (Rosenzweig et al. 1999)] and LYS7 [PDB code 1QUP (Lamb et al. 1999)], Cu chaperone CopZ from *Enterococcus hirae* [PDB code 1CPZ (Wimmer et al. 1999)], and the mercury-binding protein MerP from *Shigella flexneri* [PDB code 1AFI (Steele and Opella 1997)]; the cylinders indicate α-helices and the *arrows* indicate β-strands. The different lengths of the two MerP proteins refer to the mature and the unprocessed forms of the protein. Species name abbreviations are as follows: Bs, *Bacillus subtilis;* Cj, *Campylobacter jejuni;* DR, *Deinococcus radiodurans;* HI, *Haemophilus influenzae;* HP, *Helicobacter pylori;* NMB, *Neisseria meningitidis;* TM, *Thermotoga maritima;* Sy, *Synechocystis* sp.; APE, *Aeropyrum pernix;* Aq, *Aquifex aeolicus;* UU, *Ureaplasma urealyticum,* ENTHR, *Enterococcus hirae;* CAEEL, *Caenorhabditis elegans;* MTH, *Methanobacterium thermoautotrophicum;* Mtu, *Mycobacterium tuberculosis;* SHIFL, *Shigella flexneri;* PSEAE, *Pseudomonas aeruginosa;* STAAU, *Staphylococcus aureus.*

2000). As a result, the sequences coding for multiple HMA domains are often located next to each other (Jordan et al. 2000). While transcriptional coupling of the Cu chaperone and Cu-ATPase genes is biologically plausible, these (predicted) operons exhibit a surprising diversity of gene order and the number of consecutive HMA domains (Jordan et al. 2000).

Here we present an evolutionary analysis that supports the notion that Cu chaperones and HMA domains of Cu-ATPases represent ancient and distinct lineages that have evolved largely independently since their initial radiation. Considered together with the genomic organization of the corresponding genes, these results suggest independent formation of Cu chaperone–Cu-ATPase operons in different phylogenetic lineages of prokaryotes, probably due to a strong selective pressure toward their coexpression.

Methods

The multiple alignment of the HMA domains of Cu chaperones and Cu-ATPases was constructed on the basis of a ClustalW (Higgins et al.

B

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COPZ_ENTHR  (68)   1  -MKQEFSVKGMSCNHCVARIEEAVGRISGVKKVKVQLKKEKAVVKFDEANVQATEICQAINELGYQAEV   68  1CPZA
COPA_ENTHR (727)   5  TKMBTFVITGMTCANCSARIEKELNEQPGVMSATVMLATEKASVKYTD--TTTERLIKSVENIGYGAIL   71  290642

ATX1_YEAST  (73)   3  EIKHYQFNVVMTCSGCSGAVNKVLTKLEPDVSK-IDIS-LEKQLVDVYTTLPYDFILEKIKKTGKEVRS  69  1CC7
LYS7_YEAST (249)   5  DTYEATYAIPMHCENCVNDIKACLKNVPGINSLNFDIEQQIMSVESSV---APSTIINTLRNOGKDAII  70  1QUPA
ATU2_YEAST(1004)   1  MREVILAVHGMTCSACTNTINTQLRALEGVTKCDISLVTNECQVTYDN-EVTADSIKEIIEDOGFDCEI  68  728935
                  79  TKEGLLSVQGMTCSSCVSTVTKQVEGIEGVESVVVSLVTEECHVIYEPSKTTLETAREMIEDOGFDSNI 147  728935

CUC1_CAEEL  (69)   1  -MTQYVFEMGMTCNGCANAARKVLGKL-GEDKIKIDDINVETKKITVTTDLPASDVLEALKKTGKEIKQ  67  4165309
CUA1_CAEEL(1238)  46  VQETMLBIRGMTCQSCVKNIQDVIGAKPGIHSIQVNLKEENAKCSFDTTKWTAEKVAEAVDDMGFDCKV 114  9367167
                 129  IRRAIVSIEGMTCHACVNNIQDTVGSKDGVKIVVSLEQKQGTVDVMSEKMWNGESVAESIDDMGFDCKL 197  9367167
                 249  LEKCTBAVEGMTCASCVQYIBRNISKIEGVHSIVVALIAAKAEVIYDGRVTSSDAIREHMTGELGYKAT 317  9367167

ATOX_HUMAN  (68)   1  MPKHEFSVD-MTCGGCAEAVSRVLNKLGGVKYDIDLPNKKVCIESEHSMDTLLATLKKTGKTVSTLGLE  68  4757804
CCS_HUMAN  (274)  11  LCTLBFANQ-MTCQSCVDAVRKSLQGVAGVQDVEVHLEDQMVLVHTTL---PSQENQALLEGTGRQAVL  75  4826665
                 129  LHVHQVGDLTVNCNSCGNHFNPDGASHGGPQDSDRHRGDLGNVRADADGRAIFRMEDEQLKVWDVIGRS 197  4826665
AT7B_HUMAN(1465)  57  VATSTVRILGMTCQSCVKSIEDRISNLKGVISMKVSLEQDSATVKYVPSVVCLQQVCHQIGDMGFEASI 125  1703455
                 142  EAVVKERVEGMTCQSCVSSIEGKVRKLQGVVRWKVSLSNQEAVITYQPYLIQPEDLRDHVNDMGFEAAI 210  1703455
                 256  VVTLQLRIDGMNCKSCVLNIBENIGQLLAVQSIQVSLENKTAQVKYDPSCTSPWALQRAIEALPPGNFK 324  1703455
                 360  CSTPLIAIAGMTCASCVHSIBGMISQLBGVQQISVSLABGTATVLYNPSVISPEBIRAAIEDMGFEASV 426  1703455
                 487  PQKQFLQIKGMTCASCVSNIERNLQKEAGVLSVLVALMAGKAEIKYDPEVIQPLEIAQFIQDLGFEAAV 555  1703455
                 563  DGMIELTIDGMTCASCVHNIESKLTRTMGITYASVALATSKALVKFDPEIIGPRDIIKIIEEIGFHASL 631  1703455
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Stand-alone copper-transport ATPases
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ATCU_ECOLI (834)   2  SQTIDLTLDGLSCGHCVKRVKESLEQRPDVEQADVSI--TEAHVTGTA---SAEQLIETIKQAGYDASV  65  2493016
                  98  DDSQQLLISGHSCASCVTRVQNALQSVPGVTQARVMLAERTALVMGSA---SPQDLVQAVEKAGYGAEA 163  2493016
MTH1535    (790)   1  MKRITIRIGGMSCAACALKIEEALRKLDGIRDAANVLVEGKVSVEYDPRRVDLSDVEAAIEDAGYTVLN  69  2622654
                  70  -ENIAMAVMGGMSCAMCVQKIESALRGLEGVSNATVNLAAEKAYISYNPSLTSVEDLKRTVEDLGYTVRG 137  2622654
Rv0092_Mtu (761)  13  VQRIQLRISGMSCSGCSACAHRVESTLNKLPGVRAAVNFGTRVATIDTSEA--VDAAALCQAVRRAGYQADL  79  1405763
Rv0103c_Mtu(752)  14  VRRIRLDVLGHSCSACSASRVETKLNKIPGVRASVNFATRVATIDAVGM---AADEICGVVEKAGYHAAP  79  1405774
```

Periplasmic mercury-binding proteins
```
MERP_SHIFL  (72)   2  TQTVTLAVPGMTCAACPITVKKALSKVEGVSKVDVGFEKREAVVTFDDTKASVQKLTKATADAGYPSSV  70  1AFI
MERP_PSEAE  (91)  21  TQTVTLSVPGMTCSACPITVKKALSKVEGVSKVDVTFETRQAVVTFDDAKTSVQKLTKATEDAGYPSSV  89  127008
```

Mercuric reductase
```
MERA_SHIFL (564)   1  --MSTLKITGMTCDSCAVHVKDALEKVPGVQSADVSYAKGSAKLAIEV-GTSPDALTAAVAGLGYRATL  66  126994
MERA_PSEAE (561)   1  --MTHLKITGMTCDSCAAHVKEALEKVPGVQSALVSYPKGTAQLAIVP-GTSPDALTAAVAGLGYKATL  66  126988
MERA_STAAU (547)   4  -NSYKIPIQGHITGTGCEEHVTEALEQA-GAKDVSADFRRGEAIFELSD--DQIEKAKQNISAAGYQPGE  68  126995
```

STRUCTURE

Fig. 1. (continued)

1996) alignment and corrected according to the results of gapped BLAST (Altschul et al. 1997) comparisons and the HMA domain three-dimensional structure (Lamb et al. 1999, 2000). Phylogenetic trees based on this alignment (69 sites) were generated using both parsimony and distance-based methods. The maximum-parsimony method was employed using the PAUP* v4.0 (Swofford 1998) program. The full heuristic search option was used with tree-bisection–reconnection branch swapping and random stepwise addition (10 replicates) of sequences. The same search options were used to perform 100 bootstrap replicates. A permutation-tail probability (PTP) test (Archie 1989a, b; Faith 1990) (100 replicates) was performed using PAUP* v4.0 with the same search options as above. The neighbor-joining trees (Saitou and Nei 1987) were built using distances calculated with the Dayhoff PAM matrix as implemented in the PROTDIST and NEIGHBOR program of the PHYLIP package (Felsenstein 1996). The SEQBOOT program of PHYLIP was used to generate 1000 bootstrap replicates of the multiple alignment which were analyzed using the same program, and the majority-rule consensus tree was produced using the CONSENSE program.

Partially resolved trees (Fig. 4) that represent distinct hypotheses concerning HMA domain evolution used were generated using the MacClade v3.01 (Maddison and Maddison 1992) program. These trees were imported into PAUP* and used as topological (backbone) constraints for neighbor-joining searches, with the PAM distances described above, to generate two alternative hypothesis phylogenies. A total of three phylogenies (1, the best neighbor-joining phylogeny; 2, hypothesis 1 phylogeny; and 3, hypothesis 2 phylogeny) were compared using three phylogenetic reconstruction methods: distance-based, parsimony, and maximum likelihood. The Fitch–Margoliash (1967) algorithm implemented in the DAMBE program (Xia 2000) was used to perform two distance-based tests, Bartlett's (Zar 1996) and Newman–Kuels (Zar 1996), comparing the three alternate topologies. Three parsimony-based tests implemented in PAUP* were also used to do so: the Kishino–Hasegawa (1989) test, Templeton (1983) (Wilcoxon signed-ranks) test, and winning-sites (sign) test (Prager and Wilson 1988). The TREEPUZZLE program (Strimmer and von Haeseler 1996) was used to compare the three alternate phylogenies using the Kishino–Hasegawa (1989) test based on maximum likelihood.

Neighbor-joining, parsimony, and maximum-likelihood methods were all used to reconstruct phylogenies based on alignments of Cu chaperone, Cu-ATPase HMA domain, and ATPase sequences of six operons (Fig. 2) that contain both Cu chaperones and Cu-ATPases. The results of the neighbor-joining analysis, implemented in PHYLIP and based on PAM distances as described above, are shown in Fig. 5. Phylogenetic comparisons between the Cu chaperone tree and the Cu-ATPase HMA domain tree were done using the same procedures described above for the entire data set.

Stem–loop structures were predicted using the GCG (Madison, WI) program StemLoop with default parameters. Six hundred bases, beginning with the start codon, were analyzed for each sequence.

Results

Stand-Alone HMA Domains as Cu Chaperones

Heavy metal-associated (HMA) domains in human Cu-ATPases (Wilson and Menkes disease proteins), yeast and human Cu chaperones for superoxide dismutase, and bacterial periplasmic mercury-binding proteins (HgBPs) show significant sequence and structural similarity as well as similar Cu-binding properties (Harrison et al. 1999; Harrison et al. 2000; Rosenzweig and O'Halloran 2000) (see Fig. 1). These domains consist of 65–70 amino acid residues that form a compact structure of two α-helices and four β-strands with conserved metal-binding Cys residues located between the first α-helix and the first β-strand (Gitschier et al. 1998; Lamb et al. 1999; Rosenzweig et al. 1999; Steele and Opelia 1997; Wimmer et al. 1999). The yeast Cu chaperone for superoxide dismutase, Lys7, has an additional domain that is apparently involved in its interaction with this enzyme (Eisses et al. 2000; Hall et al. 2000; Srinivasan et al. 1998). These observations indicate that stand-alone HMA domains are capable of sequestering and trafficking intracellular Cu. We therefore predict that all microbial stand-alone HMA domains are Cu chaperones; it remains to be determined if they deliver Cu to any enzyme other than Cu-ATPase.

Homology and Evolutionary History of the HMA Domains in Cu Chaperones and Cu-ATPases

The alignment of HMA domains from Cu chaperones, Cu-ATPases, and HgBPs (Fig. 1) shows absolute conservation of the two metal-binding Cys residues and good conservation of three glycine residues that are located at the end of the first β-strand and in the turns between the first α-helix and the second β-strand and between the second α-helix and the fourth β-strand, respectively (Lamb et al. 1999, 2000). Sequence and structure conservation of the HMA domains in Cu chaperones, Cu-ATPases, and HgBPs (Rosenzweig and O'Halloran 2000) clearly indicates that all these domains share a common ancestor. Given this homology, we sought to investigate, by means of phylogenetic analysis, how the HMA domains evolved in their disparate genic contexts (Table 1 and Fig. 2). Primarily, we wished to ascertain whether Cu chaperones represent a distinct evolutionary lineage separate from the HMA domains of Cu-ATPases. In addition, we addressed the question whether Cu chaperones and their corresponding coregulated Cu-ATPases have evolved as linked units.

Except for several conserved residues and the overall structural conservation, the HMA domain sequences from different organisms exhibit considerable variability (Fig. 1). This variability, together with the small size of the HMA domains, resulted in a low statistical support for most internal branches of the phylogenetic trees reconstructed from the HMA alignment. Nevertheless, the general topology of the HMA phylogeny, which shows separate Cu chaperone and Cu-ATPase groups (Fig. 3), seemed to be best compatible with the notion that Cu chaperones and the HMA domains of Cu-ATPases represent distinct phylogenetic groups. Given the preponderance of low bootstrap values, we sought to ensure that the alignment did in fact contain some reliable phylogenetic signal. A permutation-tail probability test (Archie 1989a, b; Faith 1990) indicated that the HMA alignment does include a hierarchical structure ($P < 0.01$) which probably represents a phylogenetic signal.

Given the existence of hierarchical structure in the HMA alignment, comparisons of entire phylogenies (as opposed to individual branches) were used to test specific hypotheses concerning Cu chaperone and HMA domain evolution. Phylogenies that represented distinct evolutionary scenarios were generated using neighbor-joining searches with constraint trees (Fig. 4). The alternative phylogenies reflected two distinct hypotheses: (1) Cu chaperones represent a distinct evolutionary lineage separate from the HMA domains of Cu-ATPases, and (2) each Cu chaperone has evolved via a duplication of the adjacent HMA domain of a Cu-ATPase. Subsequent to phylogenetic reconstruction, the phylogenies corresponding to these alternative hypotheses were compared with the best overall neighbor-joining phylogeny using six tests based on three modes of phylogenetic reconstruction: distance based, parsimony, and maximum likelihood (Table 2). The hypothesis 1 tree was not significantly different from the best tree in five of six of these tests, and both these trees were consistent with the scenario whereby Cu chaperones and HMA domains of Cu-ATPases represent distinct and ancient evolutionary lineages. The phylogeny that corresponds to hypothesis 2 is the longest of the three trees. In all six of the tests employed, both the best tree and the hypothesis 1 tree were significantly shorter ($P < 0.05$) than the hypothesis 2 tree. Thus the phylogenetic signal that exists in the data appears to favor the hypothesis of an ancient radiation of Cu chaperones and the HMA domains of Cu-ATPases over the alternative hypothesis whereby Cu chaperone genes have repeatedly evolved via duplication of the HMA domain-encoding sequences in different lineages.

Having established that Cu chaperones and Cu-ATPases represent distinct and ancient evolutionary lineages, we sought to examine the subsequent evolution of the chaperone–ATPase operons. To this end, six operons (Fig. 2) that contain both Cu chaperones and Cu-ATPases were analyzed phylogenetically. Separate phylogenetic reconstructions were performed for Cu chaperones, HMA domains of Cu-ATPases, and the ATPase domains themselves, and the topologies of the resulting trees were compared (Fig. 5). If these operons have evolved as linked units from an ancestral proto-operon, then trees reconstructed from these three domains should be congruent. Trees reconstructed based on the HMA

Table 1. Copper-dependent proteins encoded in complete genomes

Organism[a]	Copper chaperones (stand-alone HMA domains)[b]	Cu-ATPases (No. of N-terminal HMA domains)	Total No. of encoded HMA domains	Copper-dependent enzymes[c]				
				Cu,Zn-superoxide dismutase	Heme/copper-type cytochrome oxidase subunits I and II	Multi-copper oxidases	Amine oxidase	Urate oxidase
Bacteria								
Aquifex aeolicus	aq_1840a[d]	aq_1445 (1)	2	aq_238 aq_1050	aq_2190 aq_2191 aq_2192	aq_1130	—	—
Bacillus subtilis	**YvgY**	**YvgX** (2) YvgW (1)	4	YojM	CtaC CtaD QoxA QoxB	CotA	—	YunL
Campylobacter jejuni	**Cj1162c**	**Cj1161c** (1) Cj1155c (2)	4	—	Cj1490c	Cj1516	—	—
Deinococcus radiodurans	**DR2452**[e]	**DR2453** (2)[e]	3	DR1546	DR2619 DR2620	—	DRB0101[f]	DR1160
Escherichia coli	—	YbaR (2)[g]	2	SodC	CyoA CyoB	YacK SufI	TynA	—
Haemophilus influenzae	**HI0292** **HI0291** HI1050	**HI0290** (1)	4	—	—	HI0733	—	—
Helicobacter pylori	**HP1073**	**HP1072** (1)	2	—	HP0144	—	—	—
Ureaplasma urealyticum[g,h]	**UU202**[h]	**UU203**[h] (0)	1	—	—	—	—	—
Mycobacterium tuberculosis	—	Rv0092 (1) Rv0103c (1)	2	Rv0432	Rv3043c Rv2200c	Rv0846c	—	—
Neisseria meningitidis	NMB1271	NMB1325 (1)	2	NMB1398	NMB1725	NMB1623	—	—
Rickettsia prowazekii	—	—	0	—	RP405 RP406	—	—	—
Synechocystis sp.	ssr2857	sll1920 (1) slr0798 (1) slr1950 (1)	4	—	slr1136 slr1137 sll0813 slr2082	—	—	—
Thermotoga maritima	TM0320	TM0317 (1)	2	—	—	—	—	—
Archaea								
Methanobacterium thermoautotrophicum	—	MTH1535 (2)	2	—	—	—	—	—
Archaeoglobus fulgidus	AF0346	AF0473 (1+1)[i]	3	—	—	—	—	—
Aeropyrum pernix	APE0009	APE1454 (1)	2	—	APE0792 APE0793 APE1623 APE1720	—	—	—
Eukaryotes								
Saccharomyces cerevisiae	LYS7 ATX1	CCC2 (2) PCA1 (1)	5	SOD1	COX1 COX2	FET3 FET5 YDR506c	—	—
Caenorhabditis elegans	CUC-1 Y76A2A.3	CUA-1 (3)	5	C15F1.7 F55H2.1 ZK430.3	COX1 COX2	F21D5.3	—	—

[a] Only organisms that have at least one copper-dependent protein are listed. We have not found Cu chaperones, Cu-ATPases, or any Cu-dependent enzymes encoded in the genomes of *Mycoplasma genitalium, Mycoplasma pneumoniae, Borrelia burgdorferi, Treponema pallidum, Chlamydia trachomatis, Chlamydia pneumoniae, Methanococcus jannaschii, Pyrococcus horikoshii,* and *Pyrococcus abyssi.*

[b] Proteins are listed according to their gene names in the complete genome sequences, which can be used to retrieve the sequences from the NCBI protein database (http://www.ncbi.nlm.nih.gov/Entrez/protein.html). A dash indicates the absence of the corresponding gene in the complete genome, based on protein sequence comparisons using the Clusters of Orthologous Groups (http://www.ncbi.nlm.nih.gov/COG) approach. This does not necessarily mean the absence of the corresponding function, which might be carried out by a nonorthologous protein, e.g., Fe,Mn-dependent superoxide dismutase. See Koonin et al. (1998) and Tatusov et al. (1997, 2000) for more details. Cu chaperones and Cu-ATPases that are encoded by adjacent genes, presumed to comprise an operon, are in boldface.

[c] Only the Cu-dependent enzymes, commonly found in microbial genomes, are listed. Lysyl oxidase, tyrosinase, laccase, or ceruloplasmine homologues have not been found in any sequenced prokaryotic genomes.

[d] The Cu chaperone gene aq_1840a was missed in the original annotation of the *Aquifex aeolicus* genome but recognized in the course of COG-based analysis, as described previously (Natale et al. 2000).

[e] DR2452 and DR2453 genes are located on opposing DNA strands and apparently form a divergent operon (see Fig. 2).

[f] This gene is frameshifted; the corresponding protein may not be expressed in an active form.

[g] YbaR has recently been experimentally characterized and renamed CopA (Rensing et al. 2000).

[h] *Ureaplasma urealyticum* genome encodes a Cu chaperone (UU202), but the adjacent P-type ATPase (UU203) lacks an HMA domain; this could be either a sequencing artifact or an indication of specific interaction between UU202 and UU203 (see text for more details).

[i] Predicted Cu-ATPase AF0473 contains an HMA domain at its N terminus and another one at its C terminus.
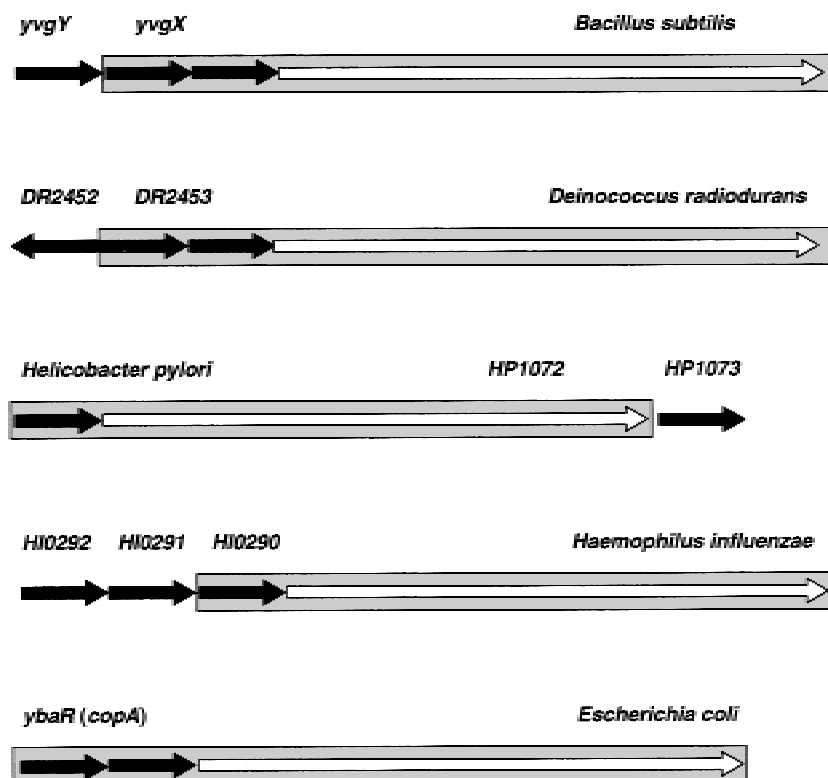
Fig. 2. Operon organization of HMA domains in Cu chaperones and Cu-transporting ATPases. The heavy metal-associated (HMA) domains are indicated by *short black arrows;* the ATPase domains are indicated by *white arrows.* The complete sequences of Cu-transporting ATPases including HMA and ATPase domains are *boxed.*

domains of Cu-ATPases and the ATPases domains were essentially identical, with the exception of one short internal branch that was not well supported (Fig. 5, top). This indicates, not unexpectedly, that the HMA and ATPase domains of Cu-ATPases encoded by the same gene have evolved as a single unit. In contrast, the topology of the Cu chaperone tree differed from those of the Cu-ATPase trees (Fig. 5, bottom). For example, bootstrap-supported association of the Cu chaperones from *H. pylori* and *T. maritima* was not observed among the corresponding Cu-ATPases. Instead, the Cu-ATPase HMA domain from *H. pylori* consistently grouped with the Cu-ATPase HMA domain from *H. influenzae* (Fig. 5, bottom). Whole-tree comparison was also employed here to test further the differences between trees. Again, six tests based on three phylogenetic reconstruction methods were used to compare the Cu chaperone tree with the Cu-ATPase HMA-domain tree. Each test indicated statistically significant differences between the two topologies (Table 3). Thus, the evolutionary history of Cu chaperones appears to differ from that of their adjacent Cu-ATPases, which seems to support the counterintuitive hypothesis of de novo formation of chaperone–ATPase operons.

Discussion

Co-Occurrence of Cu Chaperones and Cu-Dependent Enzymes

In eukaryotes, the principal function of Cu chaperones is to deliver Cu to copper-dependent enzymes, such as Cu,Zn-dependent superoxide dismutase, cytochrome oxidase, and tyrosinase (Harrison et al. 1999, 2000; Rosenzweig and O'Halloran 2000). Their role in Cu detoxification seems to be minor, if any; this function is performed by metallothioneins (Dameron and Harrison 1998). In bacteria, the only experimentally characterized Cu chaperone, CopZ from *Enterococcus hirae,* has been implicated in maintaining copper homeostasis in the cell (Cobine et al. 1999; Harrison et al. 1999, 2000). A comparison of the repertoires of Cu-binding proteins encoded in each of the complete genomes shows that, as a rule, Cu chaperones are encoded only in those genomes that also encode a Cu-ATPase and at least one Cu-dependent enzyme (Table 1). The only exception seems to be *Ureaplasma urealyticum* that encodes a Cu chaperone (UU202) but no other Cu-binding proteins (Table 1). In fact, the next *U. urealyticum* gene (UU203) is in frame with UU202 and encodes a P-type ATPase that is very similar to Cu-ATPases but lacks an HMA domain. Phylogenetic analysis shows that UU202 clusters with HMA domains of Cu-ATPases, not with Cu chaperones (Fig. 3). Thus UU202 is, in effect, an ATPase HMA domain that has been separated from the rest of the ATPase gene due to a genuine nonsense mutation or a sequencing error.

Two more exceptions of the general rule, *E. coli* and *M. tuberculosis,* each encode Cu,Zn-type superoxide dismutases and heme copper-type quinol oxidases but lack a dedicated Cu chaperone gene (Table 1). The *E. coli* Cu-ATPase contains two tandem HMA domains, and the N-terminal one has been identified in *E. coli* protein ex-
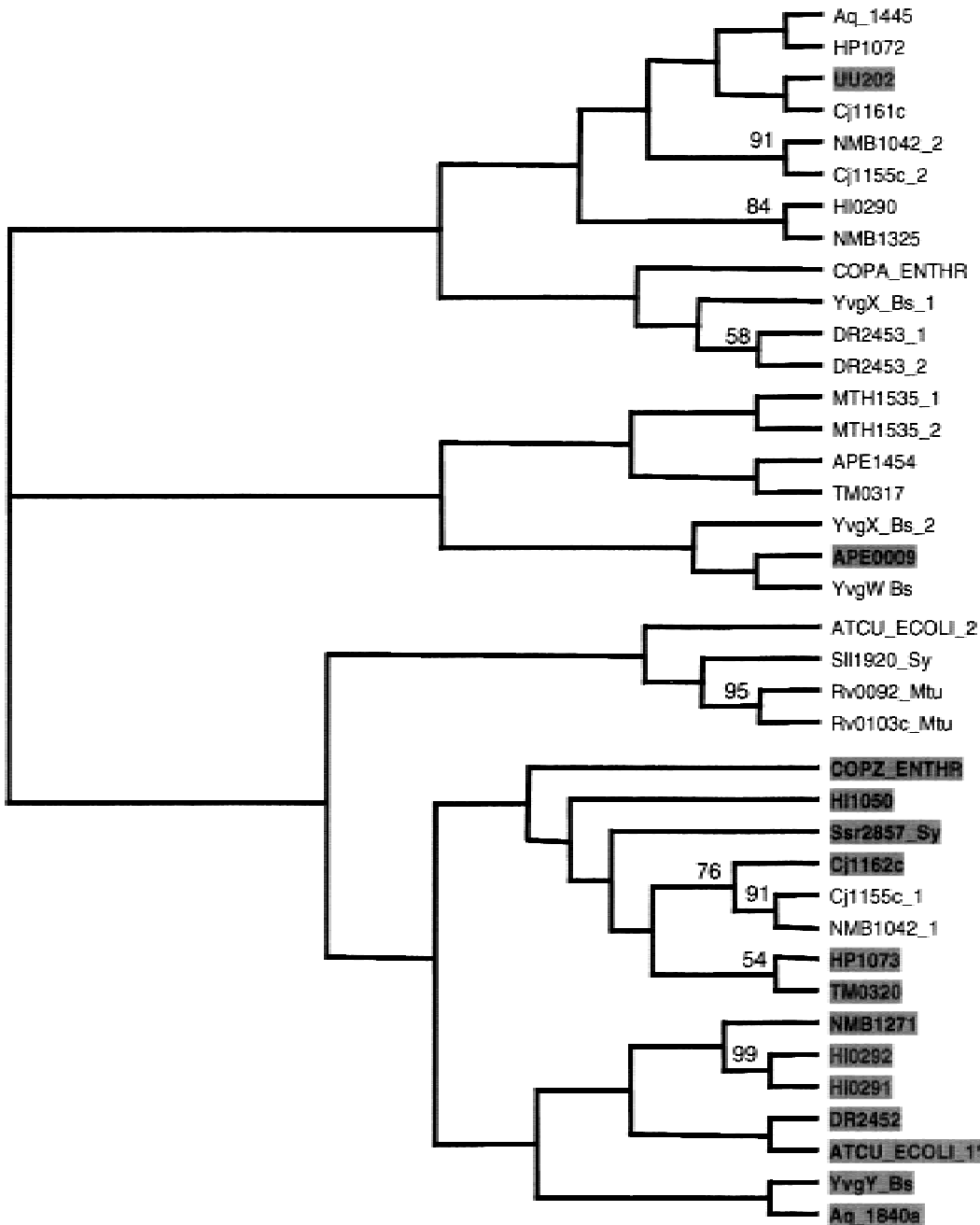
Fig. 3. Neighbor-joining phylogenetic tree of the HMA domains in Cu chaperones and Cu-transporting ATPases. The designations of the HMA domain-containing proteins are as in Fig. 1. Consecutive HMA domains from a single protein are indicated as _1 or _2, starting from the N-terminal domain. Names of stand-alone HMA domains (Cu chaperones) are *shaded*. The *E coli* N-terminal HMA domain, discussed in the text, is labeled with an *asterisk*. Bootstrap values >50% are shown.

tracts as a separate protein (Wasinger and Humphery-Smith 1998), suggesting that, in this bacterium, a Cu chaperone might be generated by proteolytic cleavage of the Cu-ATPase (Jordan et al. 2000). Alternatively, it could be formed by an early termination of transcription (or translation) of the Cu-ATPase gene. This second possibility, however, appears less likely because the region of the *copA* gene that separates the two HMA domains does not seem to form any strong stem–loop structures (data not shown). The phylogenetic data placed the N-terminal HMA domain of the *E. coli* Cu-ATPase among the Cu chaperones (Fig. 3), suggesting a relatively late fusion of this domain with an ancestral, single-HMA domain-containing ATPase. Sequence analysis of unfinished bacterial genomes showed the same organization of the Cu-ATPase gene in other members of *Enterobacteriaceae,* but not in all γ-proteobacteria (not shown), indicating that this fusion could be specific for the enterobacterial lineage.

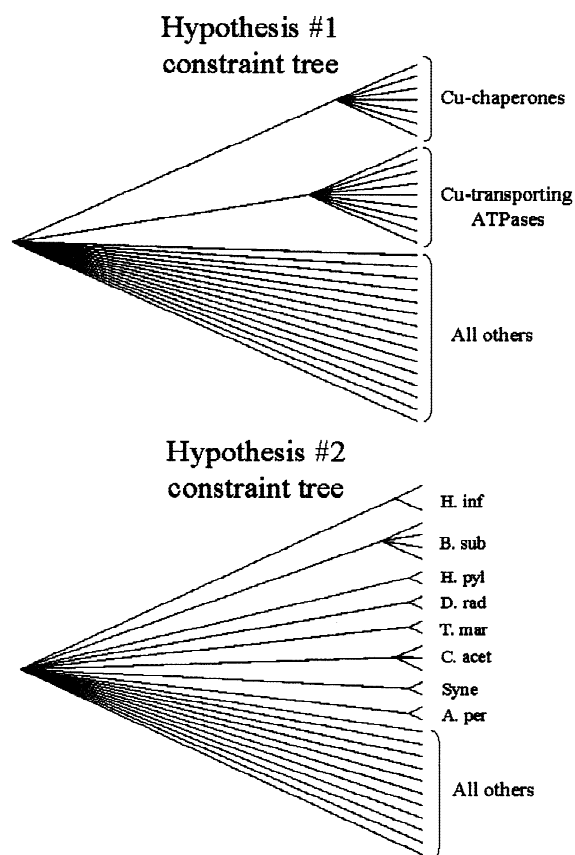A similar mechanism could have emerged in *Myco-*

Hypothesis #1
constraint tree

Cu-chaperones

Cu-transporting ATPases

All others

Hypothesis #2
constraint tree

H. inf
B. sub
H. pyl
D. rad
T. mar
C. acet
Syne
A. per

All others

Fig. 4. Unresolved topologies corresponding to hypothesis-specific constraint trees. Hypothesis 1 holds that Cu chaperones represent a distinct evolutionary lineage separate from the HMA domains of Cu-ATPases. Hypothesis 2 holds that each Cu chaperone has evolved via a duplication of the adjacent HMA domain of a Cu-ATPase.

Table 2. Statistical comparisons of the best neighbor-joining (NJ) tree with the hypothesis 1 (Hyp 1) tree and hypothesis 2 (Hyp 2) tree[a]

Distance based: Bartlett's test				
Tree	Length	Variance	F	P[b]
NJ	20.52578	0.01514	1.076	0.1670
Hyp 1	20.79250	0.01407	Best	
Hyp 2	22.82852	0.02372	1.686	0.0000

Distance based: Student–Newman–Keuls test			
Tree comparison	Diff. log (var.)	q	P[b]
NJ vs Hyp 1	0.07302	1.3558	0.3367
NJ vs Hyp 2	0.44912	8.3269	<0.001
Hyp 1 vs Hyp 2	0.52214	9.6912	<0.001

Parsimony based: Kishino–Hasegawa test				
Tree	Length	Diff.	SD	t	P[b]
NJ	1203	Best			
Hyp 1	1221	18	9.93656	1.8115	0.0745
Hyp 2	1303	100	15.83369	6.3156	<0.0001

Parsimony based: Templeton (Wilcoxon signed-ranks) test			
Tree	Length	Rank sums	Z	P[b]
NJ	1203	Best		
Hyp 1	1221	700.5 (−427.5)	1.5072	0.1318
Hyp 2	1303	1254.5 (−123.5)	5.1986	<0.0001

Parsimony based: Winning-sites test			
Tree	Length	Counts	P[b]
NJ	1203	Best	
Hyp 1	1221	32 (−15)	0.0196
Hyp 2	1303	44 (−8)	<0.0001

Maximum likelihood based: Kishino–Hasegawa test			
Tree	Log likelihood	Diff.	SE	P[b]
NJ	−5166.74	Best		
Hyp 1	−5184.07	17.33	14.37	>0.05
Hyp 2	−5348.69	181.95	39.43	<0.05

[a] Details of statistical tree comparisons are described under Methods.
[b] Approximate probability of getting a more extreme test statistic under the null hypothesis of no difference between the trees (best vs other) being compared.

bacterium tuberculosis. This organism encodes at least three Cu-dependent enzymes, superoxide dismutase (Wu et al. 1998), cytochrome oxidase, and a multicopper-type oxidase (Table 1), and would be expected to need a Cu chaperone. Nevertheless, a dedicated Cu chaperone-encoding gene is apparently missing in the *M. tuberculosis* genome. Moreover, both Cu-ATPases of *M. tuberculosis,* Rv0092 and Rv0103c, contain only one N-terminal HMA domain. As a result, generation of a Cu chaperone by proteolytic digestion of a Cu-ATPase seems unlikely because this would result in the formation of a solo ATPase domain without an HMA domain. A Cu chaperone formation via premature termination of transcription (or translation) in a Cu-ATPase gene(s) does not seem to be particularly plausible either due to the lack of predicted stem–loop structures in either Rv0092 or Rv0103c genes (data not shown). The mechanisms of copper homeostasis in *M. tuberculosis* warrant further investigation, particularly because the most commonly used antituberculin drugs, isoniazid and ethambutol, are both efficient copper chelators (Bottari et al. 2000; Hanson et al. 1981; Kozak et al. 1998).

Finally, *R. prowazekii* represents the only exception of a different kind, where the presence of the heme cop-

per-type cytochrome oxidase aa_3 is not accompanied by the presence of either a Cu chaperone or a Cu-ATPase (Table 1). Because *R. prowazekii* is an intracellular parasite, it can be speculated that Cu delivery to its cytochrome oxidase is performed by the Cu chaperone(s) of the host cell, probably in the same way that Cox17 delivers Cu to the mitochondrial cytochrome oxidase (Srinivasan et al. 1998).

Cu Chaperones and Cu-ATPases Are Coexpressed But Have Not Coevolved

Phylogenetic analysis shows that HMA domains of Cu chaperones and Cu-ATPases evolved independently of

Table 3. Statistical comparisons of the copper chaperone tree with the copper transporter HMA-domain tree[a]

		Distance based: Bartlett's test		
Tree	Length	Variance	F	P[b]
Chaperone	2.92205	0.00790	Best	
HMA	3.03620	0.02902	3.672	0.0130

		Distance based: Student–Newman–Keuls test			
Tree	Length	Variance	Diff. log (var.)	q	P[b]
Chaperone	2.92205	0.00790	Best		
HMA	3.03620	0.02902	1.30066	3.1860	0.0243

		Parsimony based: Kishino–Hasegawa test			
Tree	Length	Diff.	SD	t	P[b]
Chaperone	163	Best			
HMA	175	12	4.71543	2.5448	0.0132

		Parsimony based: Templeton (Wilcoxon signed-ranks) test		
Tree	Length	Rank sums	Z	P[b]
Chaperone	163	Best		
HMA	175	137 (–34)	2.4493	0.0143

		Parsimony based: Winning-sites test	
Tree	Length	Counts	P[b]
Chaperone	163	Best	
HMA	175	14 (–4)	0.0309

		Maximum-likelihood based: Kishino–Hasegawa test		
Tree	Log likelihood	Diff.	SE	P[b]
Chaperone	–866.31	Best		
HMA	–883.46	17.15	8.74	<0.05

[a] Details of statistical tree comparisons are described under Methods.
[b] Approximate probability of getting a more extreme test statistic under the null hypothesis of no difference between the trees (best vs other) being compared.

each other, rather than as a single unit (Figs. 3 and 4 and Tables 2 and 3). Given the extensive rearrangement that is characteristic of prokaryotic genomes (Mushegian and Koonin 1996; Dandekar et al. 1998; Itoh et al. 1999), this evolutionary scenario would be expected to result in a random scatter of the chaperones and ATPases on prokaryotic chromosomes unless coexpression of these genes is selected for. While the Cu chaperone and Cu-ATPase genes are indeed located separately in several genomes, in many bacteria they seem to form operons, indicating that the two genes are coexpressed and, by inference, functionally linked (Fig. 2). Coexpression of these proteins has been reported in *C. elegans* (Wakabayashi et al. 1998), where their genes are not adjacent. In *E. hirae,* localization of a Cu chaperone and a Cu-ATPase in the same operon was attributed to their joint participation in maintaining Cu homeostasis in the cell (Cobine et al. 1999; Odermatt et al. 1992, 1994;

Wunderli-Ye and Solioz 1999). We now know that, at least in yeast cells, all Cu is chaperone-bound and the concentration of free Cu in the cytoplasm is virtually nil (Rae et al. 1999). This probably means that the functions of Cu chaperones in Cu resistance and Cu delivery are tightly linked. Under normal conditions, when extracellular Cu levels are very low, Cu-ATPases and Cu chaperones ensure Cu uptake and its delivery to Cu-dependent enzymes, respectively (Table 1). Under the conditions of excessive Cu levels, these same Cu-ATPases and Cu chaperones ensure Cu binding in the cytoplasm and its extrusion from the cell. Such a system for maintaining Cu homeostasis might involve two different Cu-ATPases for Cu uptake and extrusion. Indeed, several microbial genomes encode a single Cu chaperone and two or more Cu-ATPases (Table 1).

The juxtaposition of the Cu chaperone and Cu-ATPase in the same operon would ordinarily suggest that such operons are descendants of an ancestral operon whose gene organization had been maintained by selection. However, phylogenetic analysis fails to show congruent evolution of chaperones and ATPases, which seems to suggest that the same operonic arrangement has evolved repeatedly in different phylogenetic lineages. Operon disruption and shuffling are common in prokaryotic evolution (Itoh et al. 1999), but independent formation of the same operon in different genomes, to our knowledge, has not been documented so far. Horizontal gene transfer is widespread in prokaryotes (Doolittle 1999). Given the scattered phyletic distribution of HMA domains and the presence of several unexpected groupings in the phylogenetic tree (Fig. 3), it is a distinct possibility that horizontal transfer has contributed to the observed distribution of HMA encoding genes. However, the low bootstrap values for individual branches on the HMA tree does not allow us to provide strong support for this scenario.

HMA Domains in Mercury Resistance

One instance where the function of the HMA domain is firmly established is its involvement in mercury detoxification. This system has been extensively studied, and the function of periplasmic mercury-binding proteins (HgBPs) in delivering Hg^{2+} to the cytoplasm is well established (Lund and Brown 1987; Silver et al. 1989; Silver and Phung 1996). The presence of the HMA domains in mercury reductases, however, remains unexplained because deletion of an HMA domain was found to have no effect on the reduction of Hg^{2+} to Hg^0 (Schiering et al. 1991). Phylogenetic analysis of the HMA domains in both HgBPs and Hg reductases shows that these domains, while closely related to ones in Cu chaperones and Cu-ATPases, form a separate well-defined branch (data not shown). It appears that HgBPs have evolved from the ancestral HMA domains by pick-
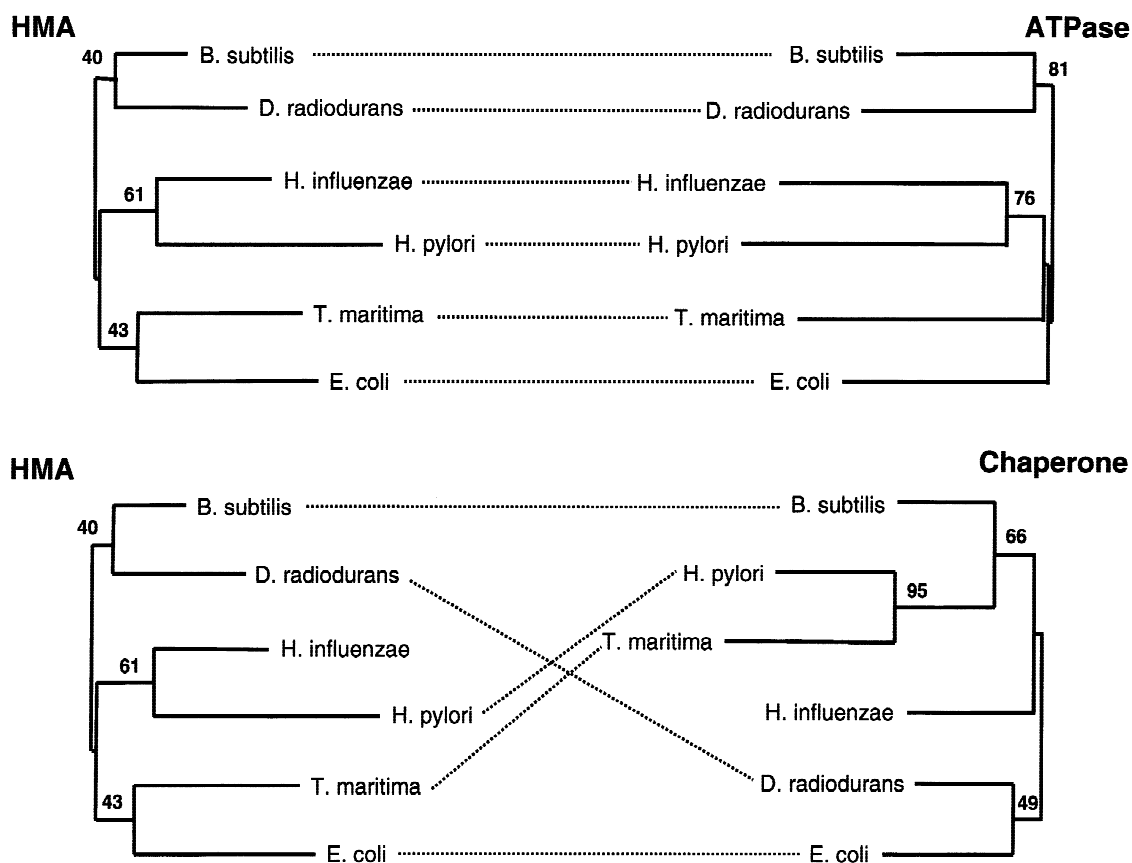
Fig. 5. A comparison of the topologies of neighbor-joining phylogenetic trees of the Cu chaperones and Cu-transporting ATPases. **Top:** A comparison of the trees for the N-terminal HMA domains and non-HMA portions of Cu-transporting ATPases. For Cu-transporting ATPases from *B. subtilis* and *D. radiodurans,* only the N-terminal domains BS_YvgX_1 and DR2453_1 were used in the HMA tree. For the Cu-transporting ATPase YbaR (CopA) from *E coli,* the N-terminal HMA domain ATCU_ECOLI_1 was used in the chaperone tree, whereas the second HMA domain ATCU_ECOLI_2 was used in HMA tree. See text for a discussion of the mechanism of Cu chaperone formation in *E coli.* **Bottom:** A comparison of the trees for HMA domains of Cu-transporting ATPases and their adjacent Cu chaperones. Bootstrap values >40% are shown.

ing up N-terminal signal sequences that allowed their export into periplasm (Fig. 1).

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