Evolution of a Heavy Metal Homeostasis/Resistance Island Reflects Increasing Copper Stress in Enterobacteria

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Accepted: February 13, 2016

Data Deposition: This project has been deposited at NCBI GenBank under the accession KC146966 and at NCBI BioProject under the accession PRJNA217802.

Abstract

Copper homeostasis in bacteria is challenged by periodic elevation of copper levels in the environment, arising from both natural sources and human inputs. Several mechanisms have evolved to efflux copper from bacterial cells, including the *cus* (copper sensing copper efflux system), and *pco* (plasmid-borne copper resistance system) systems. The genes belonging to these two systems can be physically clustered in a Copper Homeostasis and Silver Resistance Island (CHASRI) on both plasmids and chromosomes in Enterobacteria. Increasing use of copper in agricultural and industrial applications raises questions about the role of human activity in the evolution of novel copper resistance mechanisms. Here we present evidence that CHASRI emerged and diversified in response to copper deposition across aerobic and anaerobic environments. An analysis of diversification rates and a molecular clock model suggest that CHASRI experienced repeated episodes of elevated diversification that could correspond to peaks in human copper production. Phylogenetic analyses suggest that CHASRI originated in a relative of *Enterobacter cloacae* as the ultimate product of sequential assembly of several pre-existing two-gene modules. Once assembled, CHASRI dispersed via horizontal gene transfer within Enterobacteriaceae and also to certain members of Shewanellaceae, where the original *pco* module was replaced by a divergent *pco* homolog. Analyses of copper stress mitigation suggest that CHASRI confers increased resistance aerobically, anaerobically, and during shifts between aerobic and anaerobic environments, which could explain its persistence in facultative anaerobes and emergent enteric pathogens.

Key words: gene cluster, horizontal transfer, genetic module, metal resistance.

Introduction

Humans have employed copper over the last eight millennia for tools, disinfection, plumbing, manufacturing, animal husbandry, crop protection, and preservation of perishable commodities (Hobman and Crossman 2015). This usage has exposed many bacteria in the environment and animal microbiomes to unprecedented levels of copper stress. Intracellular copper stress in animal-associated Enterobacteria such as *Escherichia coli* is modulated by both chromosomal and plasmid-encoded copper trafficking and export mechanisms (Finney and O'Halloran 2003; Nies 2003; Rensing and Grass 2003). The combined activity of these systems maintains homeostasis and compartmentalization of this essential, but potentially toxic, metal. The *Es. coli* chromosome contains a monovalent copper export system, *cus* (copper sensing copper efflux system; Munson et al. 2000; Franke et al. 2003), which combines with the muticopper oxidase (MCO) and P-type ATPase of the chromosomal *cue* system (Rensing et al. 2000; Grass and Rensing 2001; Stoyanov et al. 2001) to detoxify and export copper ions from the cell during aerobic and anaerobic copper stress (Outten et al. 2001; Fung et al. 2013). The *cus* system, which is particularly important for copper resistance in growth under anaerobic conditions (Outten et al. 2001), shares sequence and functional homology with the silver-resistance system *sil* in the plasmid pMG101 (Gupta et al. 1999; Silver 2003), isolated from *Salmonella typhimurium* growing on burns treated with silver nitrate (McHugh et al. 1975). *cus* is also known to contribute to infection in

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pathogenic *Es. coli* (Huang et al. 1999) and is associated with neonatal *Cronobacter* meningitis but not other *Cronobacter* strains (Kucerova et al. 2010).

Another copper resistance gene cluster, *pco* (plasmid-borne copper resistance system), was discovered on plasmid pRJ1004 following isolation from *Es. coli* growing in Australian piggery effluent on feedlots where copper supplements were employed in growth stimulation and antibiotic roles (Tetaz and Luke 1983). pRJ1004 confers a 3-fold increase in copper resistance over endogenous homeostasis mechanisms to *Es. coli* cells grown on nutrient agar supplemented with $CuSO_4$ (Rouch et al. 1985). Analyses of pRJ1004 in *Es. coli* revealed that inducible copper resistance on this plasmid (Rouch et al. 1985) is mediated by a cluster of seven *pco* genes (Bryson et al. 1993; Brown et al. 1995), which are homologous to the *cop* system from plasmid pPT23D in *Pseudomonas syringae* and the genomic and plasmid-encoded systems in *Xanthomonas* spp. (Cooksey 1987; Lee et al. 1994; Mellano and Cooksey 1988a; Voloudakis et al. 1993) and contribute to inducible copper resistance under a range of copper stress conditions (Brown et al. 1995; Rouch and Brown 1997).

Here we describe physiological, genomic, and phylogenetic evidence for a transposable element-flanked island of 19 genes that includes adjacent *cus* and *pco* clusters, which we call the Copper Homeostasis and Silver Resistance Island (CHASRI). In order to better understand how this compound resistance mechanism emerged, we investigated the patterns of CHASRI sequence diversification and the functional advantage it confers. Phylogenetic analyses suggested the CHASRI was derived from a single event involving the linkage of the two copper resistance mechanisms, likely in a close relative of *Enterobacter cloacae*. The constituent resistance mechanisms (*cus* and *pco*) are themselves the products of associations of multiple pre-existing modules that largely correspond to functional subcomplexes within the copper export systems (fig. 1 and supplementary table S1, Supplementary Material online). Since its assembly, CHASRI has realized a spotty dispersal by horizontal transfer among emergent pathogens in Enterobacteria and other bacteria families under both natural and anthropogenic copper stress. We find that CHASRI confers copper tolerance aerobically, anaerobically, and during transitions between aerobic and anaerobic growth. Molecular dating of CHASRI sequences suggested that increases in anthropogenic copper have influenced its dispersal in bacterial pan-genomes.

Materials and Methods

Plasmid Sequencing

Using the *pco* operon as a starting point, a polymerase chain reaction (PCR)-based gene-walking strategy was used to

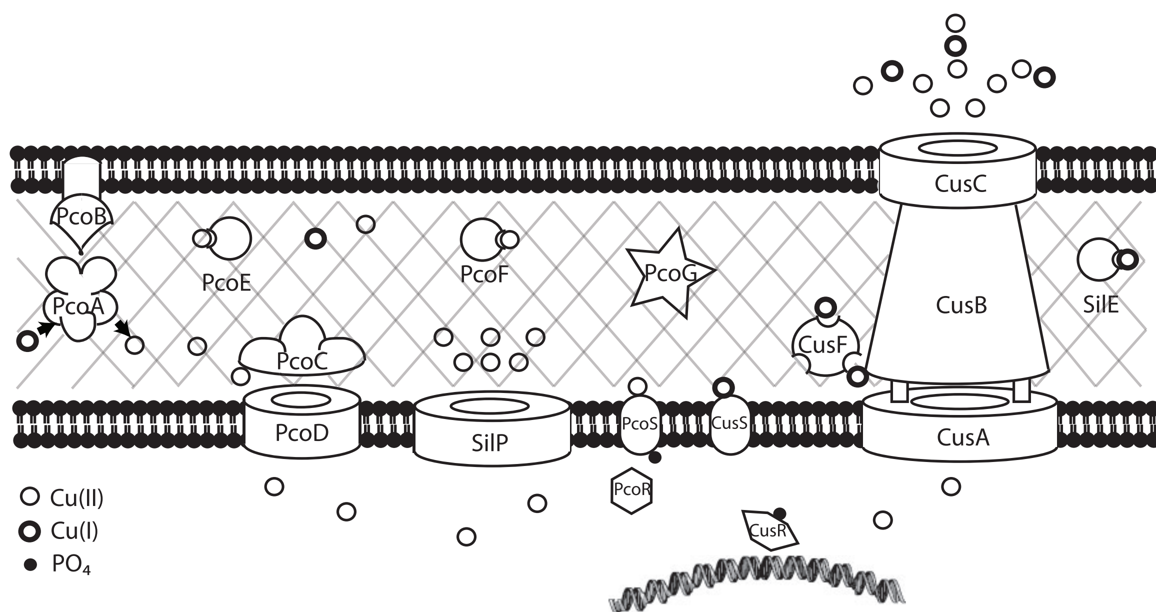


Fig. 1.—Proposed integrated function of CHASRI module genes in homeostasis. The three major mechanisms of copper detoxification encoded in the CHASRI are the periplasmic detoxification pco/CopABCD system, the P-ATPase (silP) involved in efficient cytoplasmic detoxification, and the cus/sil tricomponent cation efflux mechanism. The putative function of pcoA = multicopper oxidase, pcoB = outer membrane transport, pcoC = periplasmic metal binding, pcoD = inner membrane transport, pcoE = periplasmic metal binding, pcoF = periplasmic metal binding, pcoG = M23 metallopeptidase, cusA = RND efflux pump (inner membrane), cusB = RND efflux pump (periplasm), cusC = RND efflux pump (outer membrane), cusF = periplasmic metal binding, silE = periplasmic metal binding. DUF411 and RS modules (pcoRS and cusRS) involved in regulatory processes are not shown. Homology with other study systems is detailed in supplementary table S1, Supplementary Material online.

recover over 17,000 bp of plasmid pRJ1004 (GenBank accession X83541.1) upstream of *pcoA*. Twenty-nine overlapping primer sets were used to generate sequences using an ABI 3730 High-Throughput DNA Sequencer, which were then assembled manually (GenBank accession # KC14966). This region was then searched for predicted open reading frames (ORFs) using NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html, last accessed March 2, 2016). The contents of the pRJ1004 plasmid were further investigated using an Illumina HiSeq strategy. Plasmid DNA was sheared via sonication using a Bioruptor (Diagenode) at high setting for 15 min in cycles of 30 s on and 30 s off. Library preparation was carried out as previously described (Gibbons et al. 2009) with the following modifications: *i* adapter ligated plasmid DNA was run out on a 2.5% agarose gel (Fisher's Nucleic Acid Recovery Agarose) in 80% glycerol loading buffer at 80 V for 2 h and *ii* the library was amplified for 12 cycles of PCR. The final library was quality controlled for integrity, size, concentration, and molarity on a Bioanalyzer (Agilent). The library was run, with other samples for 57 cycles, on a single lane of an Illumina HiSeq 2000 at the Vanderbilt Genome Sciences Resource (NCBI BioProject # PRJNA217802).

Detection of Related Clusters and Plasmids

Using copper cluster-associated amino acid sequences as queries, we searched for homologous protein models in a database of 1,150 prokaryotic proteomes (supplementary table S2, Supplementary Material online), including both plasmids and chromosomes, using BLASTP (Altschul et al. 1997), retaining sequences that were greater than 45% similar at the amino acid level and between 40% and 150% the length of the query. Protein families were delimited using OrthoMCL (version 1.4) (Li 2003) at an inflation value of 2.0. Clustering of *cus* and *pco* gene homologs was inferred when two or more homologs were separated by no more than six intervening genes as described previously (Campbell et al. 2012).

Similarity between the pRJ1004 genome and other plasmids was investigated with a mapping strategy in which 51 bp reads were used as BLASTN queries against 2,556 sequences of the plasmid database at NCBI (supplementary table S3, Supplementary Material online), and locations with greater than 90% identity and overlap were considered homologous to the query.

Phylogenetic Methods

Gene family amino acid sequences were aligned using mafft (version 6.847) (Katoh et al. 2002; Katoh and Toh 2008), and maximum likelihood (ML) analysis was performed in RAxML (version 7.2.8) (Stamatakis 2006) under the PROTGAMMAJTT model of amino acid substitution. Phylogenetic analysis, which included additional sequences retrieved from NCBI nr via BLASTN, was also performed on nucleotide sequences of the region corresponding to *silE*, *cusSRCFBA*, and *silP* in RAxML under the GTRCAT model of evolution. Support for amino acid and nucleotide analyses was assessed with 100 bootstrap replicates in RAxML. A bacterial species phylogeny was constructed with a database-wide alignment of RNA polymerase, beta prime subunit subjected to 100 neighbor-joining bootstrap replicates in PAUP* 4.0b (Swofford 2003). Correlations between gene family phylogenies were measured by multiplying the proportions of shared reciprocal best-BLAST hits (rbh) between pairs of proteins out of the total number of rbh genetic element (chromosome or plasmid) pairs for each protein in the comparison. We performed a molecular clock analysis on the nucleotide alignment in BEAST v. 1.7.1 (http://beast.bio.ed.ac.uk/beast, last accessed March 2, 2016) with the following assumptions: An uncorrelated relaxed molecular clock, lognormal rate distribution, HKY model of nucleotide substitution with four gamma categories, normally distributed priors, a Yule process tree model, and UPGMA starting tree. *Shewanella putrifaciens* strain CN32, isolated from shale sandstone 230 m below ground level in New Mexico (Fredrickson et al. 1997), has been estimated to be 2,060 to 3,500 years removed from contact with the surface given the local rate of ground water movement (Walvoord et al. 1999). We used a groundwater age estimate of 2,060 years as a minimum divergence time (normally distributed with a standard deviation of 100 years) between the deep subterranean *S. putrifaciens* CN-32 and *Es. coli A2363*. We separately applied the minimum divergence time to the split between the deep subterranean and estuary *Shewanella* species ANA-3. We ran eight independent Markov chain Monte Carlo (MCMC) simulations for ten million generations each and assessed convergence of log-likelihood values in Tracer, discarding the first 10–40% of generations. We combined trees in LogCombiner version 1.7.1 and summarized 95% highest posterior density range distributions for node ages in TreeAnnotator version 1.7.1. Effective sample sizes exceeded 200 for all parameters. A lineage through time plot was computed in the APE package v3.0-4 (http://ape-package.ird.fr, last accessed March 2, 2016) from the maximum clade credibility tree drawn in TreeAnnotator v1.7.1 using median node heights.

Copper Challenge Experiments

We compared the copper tolerance of cells containing either the entire CHASRI gene cluster, as found on plasmid pRJ1004 (Tetaz and Luke 1983), or part of the cluster, from constructed plasmid pCOIV239-B1 (Munson et al. 2000), by growth assays on solid media and by determining growth rates in liquid media. As described in the Results section, the incomplete CHASRI cluster in pCOIV239-B1 contains *pcoABCDRSE* but is missing *pcoFG* and the plasmid-encoded region of *cus*. The parent vector for pCOIV239-B1 is derived from the pSC101 family, so the low copy number (Cohen and Chang 1977) should be comparable with that of a large, naturally

occurring plasmid-like pRJ1004. Plasmids pRJ1004 and pCOIV239-B1 were transformed into *Es. coli* strains BW25113 and W3110 and successful transformants were selected by plating on lysogeny broth (LB) media (Difco) plus agar (BD) with the appropriate selection agent (170 mg/L Chloramphenicol for pCOIV239-B1, 7.5 mM $CuSO_4$ under aerobic growth in BW25113 and 2 mM $CuSO_4$ under anaerobic growth in W3110 for pRJ1004). For aerobic challenges, LB media plus agar and a 100 mM $CuSO_4$ solution (adjusted to pH 7.0 with triethanolamine to avoid precipitation and metal complexation; Magyar and Godwin 2003) were combined to make 0–50 mM $CuSO_4$ solid media in nontreated 96-well plates (BD). A 5 ml starter culture of each construct in LB media was grown with shaking at 37 °C for 6 h and 5 µl of culture was used to inoculate designated wells on each plate. The plates were incubated overnight at 37 °C. After 12 h, the wells were examined and any colony growth was considered successful tolerance of the included copper level (Pontel and Soncini 2009). In anaerobic copper challenge experiments, LB media was purged of oxygen through three cycles of vacuum evacuation/nitrogen replacement and transferred to a glove bag (I2R Model X-37-37) purged with nitrogen. Starter cultures in LB media of each construct were transferred into a glove bag and used to inoculate test tubes of 5 ml LB media. The test tubes were sealed with rubber septa and incubated overnight at 37 °C. LB media plus agar and a 100 mM $CuSO_4$ solution (adjusted to pH 7.0 with triethanolamine) were combined to make 0–50 mM $CuSO_4$ solid media in nontreated 96-well plates. The wells were inoculated with 5 µl aliquots from 5 ml anaerobic starter cultures in LB media, the plates were sealed inside a Desi-Vac container (Fisher) with an AnaeroPack (MGC), and the entire setup was stored at 37 °C. To ensure anaerobic growth, O_2 concentration in the sealed container was monitored with a ToxiPro O_2 sensor (Honeywell) and was shown to be below 0.1% for the duration of the experiment. After 48 h, the plates were examined to determine the highest level of copper tolerated by each construct.

Another set of experiments assessed aerobic growth following anaerobic copper challenge. LB media was purged of oxygen through three cycles of vacuum evacuation/nitrogen replacement and transferred to a glove bag purged with nitrogen. Starter cultures in LB media of each construct were taken into a glove bag and used to inoculate test tubes of 5 ml LB media. The test tubes were sealed with rubber septa and incubated overnight at 37 °C. LB media and a 100 mM $CuSO_4$ solution (adjusted to pH 7.0 with triethanolamine) were purged of oxygen through three cycles of vacuum evacuation/nitrogen replacement and transferred to a glove bag purged with nitrogen. Under anaerobic conditions, LB media and a 100 mM $CuSO_4$ solution (adjusted to pH 7.0 with triethanolamine) were combined to make 90 µl solutions of $CuSO_4$ media in nontreated conical bottom 96-well plates (Granier). The wells were inoculated with 10 µl aliquots from 5 ml anaerobic starter cultures in LB media (bringing the final sample volume to 100 µl and the final copper concentration from 0 to 10 mM), the plates were sealed inside a Desi-Vac container with an AneroPack, and the container was incubated at 37 °C. After 90 min, the plates were removed from anaerobic conditions and spun down at 2,500 rpm for 15 min in an Allegra 6R centrifuge (Beckman Coulter). The supernatant was removed and each pellet was resuspended in 100 µl LB or LB + $CuSO_4$ media. The plates were incubated at 37 °C with shaking for 10 h, with optical density readings at 600 nm taken every hour on a Synergy 2 microplate reader (Bio-Tek) as a proxy for cell number. Bacterial growth was fitted with the equation $Y = Y_M \times Y_0/((Y_M - Y_0)e^{(-k(x-x0))} + Y_0)$ (Vandermeer 2010) and growth rate constant (k) and growth lag (x_0) were compared using the extra sum-of-squares *F* test, implemented in GraphPad Prism version 5.04 for Windows (GraphPad Software, La Jolla, CA).

Results

A Multipart Resistance Island on pRJ1004 Confers Resistance to Copper during Changes in Environmental Oxygen

Targeted sequencing of the pRJ1004 plasmid in *Es. coli* K-12 revealed a Copper Homeostasis and Silver Resistance Island (CHASRI) of 19 genes, 6 of which are homologous to the genomic *Es. coli cus* cluster (*cusABFCRS*) (Munson et al. 2000) and two more that complete homology with the *sil* heavy metal export system cluster (*silE* and *silP*) (Gupta et al. 1999). The 11 remaining genes include the known *pco* gene cluster (*pcoABCDRSE*) (Brown et al. 1995) and 2 additional *pco* genes (*pcoF* and *pcoG*) transcribed together from the P_{pcoF} promoter in a copper-inducible manner and regulated by PcoRS (Munson 1997). BLAST-based mapping of Illumina short reads (fig. 2) revealed that pRJ1004 is most similar overall to plasmid Collb-P9 from *Es. coli* K12 (NC_002122) and related plasmids in *Escherichia*, *Shigella*, and *Salmonella*. The pRJ1004 reads that mapped to the CHASRI did not show any similarity with Collb-P9 or the other similar plasmids, but the island-mapped reads were very similar to isolated regions in nine unrelated plasmids ranging in size from 54 (NC_013285) to 279 kbp (NC_008573). The plasmid-encoded CHASRI in *En. cloacae* is 82.5% identical to the *icus* over the alignable region.

In order to determine potential selective advantages conferred by CHASRI, we conducted several copper challenge experiments. The maximum tolerable concentration (MTC) for two *Es. coli* strains transformed with plasmids containing complete or partial CHASRI genes was established under aerobic and anaerobic growth conditions. *Escherichia coli* strains BW25113 and W3110, wild type strains that display dissimilar copper tolerance profiles (Outten et al. 2001; Pontel and Soncini 2009), were transformed with either plasmid pRJ1004, containing all of CHASRI (Tetaz and Luke 1983), or plasmid pCOIV239-B1, containing only *pcoABCDRSE*

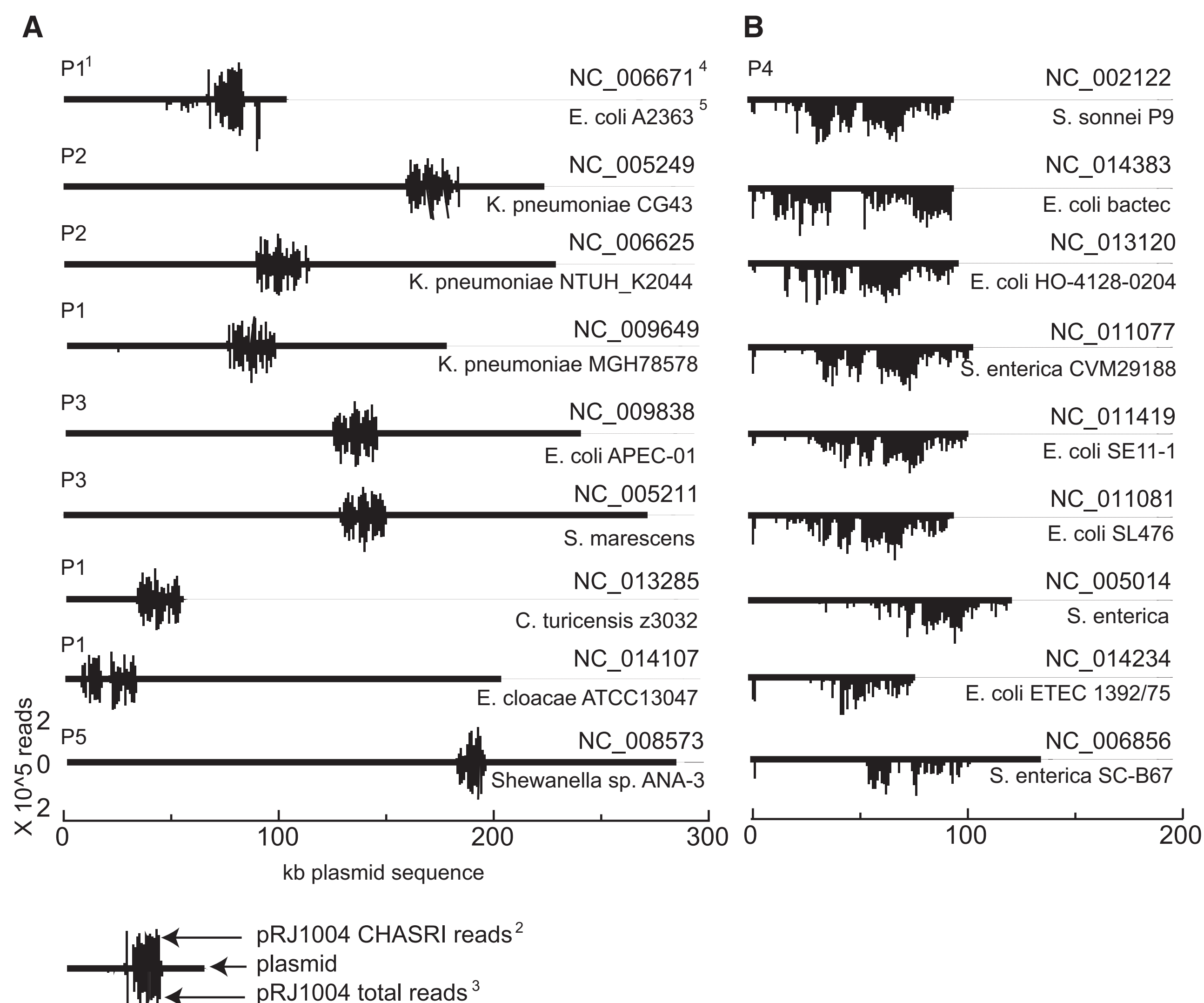


Fig. 2.—Depth of reads mapping to the plasmid database at NCBI. (*A*) The nine plasmids to which the most 50-bp Illumina HiSeq reads mapped that also mapped to the CHASRI region of pRJ1004. (*B*) The nine plasmids to which the most total pRJ1004 reads mapped. [1]Plasmid overall synteny type. [2]Depth of reads prefiltered to map to CusPco region of pRJ1004 is shown above axis. [3]Depth of total mapped reads from pRJ1004 are shown below axis. [4]GenBank accession number of plasmid. [5]Bacterial strain of plasmid isolation (where published). Reads are shown for 1,000-bp bins.

Table 1
Level of $CuSO_4$ Resistance Conferred by Constructs in *Escherichia coli*

Construct	Copper Modules Present	Aerobic Copper Tolerance[a] (mM)	Anaerobic Copper Tolerance (mM)
BW25113	*cue, icus*[b]	6.7 ± 0.3	≥50 ± 0.0
BW25113/pRJ1004	*cue, icus, pco, cus*	9.3 ± 0.3[c]	≥50 ± 0.0
BW25113/pCOIV239-B1	*cue, icus, pcoABCDRSE*	12.7 ± 0.3[c,d]	≥50 ± 0.0
W3110	*cue, icus*	15 ± 0.0	2.0 ± 0.0
W3110/pRJ1004	*cue, icus, pco, cus*	15 ± 0.0	3.0 ± 0.0[c]
W3110/pCOIV239-B1	*cue, icus, pcoABCDRSE*	15 ± 0.0	3.0 ± 0.0[c]

[a]Copper tolerance is determined by the presence of colony formation units on LB agar + $CuSO_4$ (adjusted to pH 7.0 with triethanolamine) plates. Number reported is an average of three replicates.
[b]*icus* refers to the independent chromosomally inherited *cus* cluster, whereas *cus* refers to the CHASRI-associated *cus* cluster.
[c]Significant increase in copper resistance over wild type ($P < 0.05$).
[d]Significant increase in copper resistance over pRJ1004 ($P < 0.05$).

(Munson et al. 2000), and grown on copper-containing LB media to determine resistance levels (table 1). For strain BW25513, plasmids pRJ1004 and pCOIV239-B1 conferred aerobic copper resistance 40% (P = 0.0011) and 90% (P = 0.00011) greater than wild-type copper tolerance, respectively. Under anaerobic growth conditions, none of the three constructs experienced inhibition of growth up to the highest concentration tested, 50 mM $CuSO_4$. In W3110, neither plasmid improved on the 15 mM wild-type aerobic copper resistance. Although our plasmid aerobic copper resistances were comparable with previously reported values (Tetaz and Luke 1983), our wild-type W3110 resistance consistently exceeded published levels (Grass and Rensing 2001), potentially affecting the significance of the comparison. Under anaerobic growth, both pRJ1004 and pCOIV239-B1 increased resistance to copper 50% over wild-type levels ($P < 0.01$ and $P < 0.01$, respectively). When these MTC experiments were repeated on minimal media (Fung et al. 2013) supplemented with copper, no significant difference was detected between the wild type and transformed strains across the range of copper concentration tested. Although a variety of point mutations and inversions in the W3110 genome are known to give rise to deleterious growth rate phenotypes in minimal media (Jensen 1993), similar variations in BW25113 have not been described and the lack of a significant difference between our constructs deserves further consideration.

This initial result demonstrated an advantage of CHASRI over wild-type copper resistance, but to understand the importance of the entire gene island over its constituent parts, we tested the same constructs from the MTC experiment in a growth trial that included a switch in oxygen availability during the experiment. When BW25113 cells were grown anaerobically and then switched to aerobic growth (fig. 3), there is a significantly slower growth rate for cells transformed with pCOIV239-B1, which contains only the *pcoABCDRSE* component of CHASRI, relative to those transformed with pRJ1004, which contains the intact CHASRI. We conclude that the intact CHASRI has an advantage over a partial construct under this combination of copper and oxygen stress, with one caveat: While both plasmids have low copy numbers, we cannot exclude other contributions from the different parent vectors. Compared with wild-type BW25113 cells and cells with pRJ1004, cells with pCOIV239-B1 showed a significant growth lag ($P < 0.0001$ and $P < 0.0001$, respectively) but increased growth rate ($P = 0.0420$ and $P = 0.0093$, respectively) with no copper added to the media (supplementary table S4, Supplementary Material online). When BW25113 cells were grown anaerobically in LB media containing 5 mM $CuSO_4$ then switched to aerobic growth in LB with no copper added, cells with pCOIV239-B1 again showed a significant lag in growth compared with both wild-type cells and cells with pRJ1004 ($P < 0.0001$ and $P < 0.0001$, respectively) and again demonstrated a significantly faster growth rate than wild-type cells and cells with pRJ1004 once the initial lag was overcome ($P < 0.0001$ and $P = 0.006$, respectively). Finally, when BW25113 cells were grown anaerobically in LB media containing 5 mM $CuSO_4$ then switched to aerobic growth in LB with 5 mM $CuSO_4$ added, cells with pCOIV239-B1 showed both a significant lag in growth compared with both wild-type cells and cells with pRJ1004 ($P < 0.0001$ and $P < 0.0001$, respectively) and a slower growth rate following the lag period ($P < 0.0001$ and $P < 0.0001$, respectively).

In parallel experiments using the W3110 strain, cells containing pCOIV239-B1 showed no growth lag but had a slower

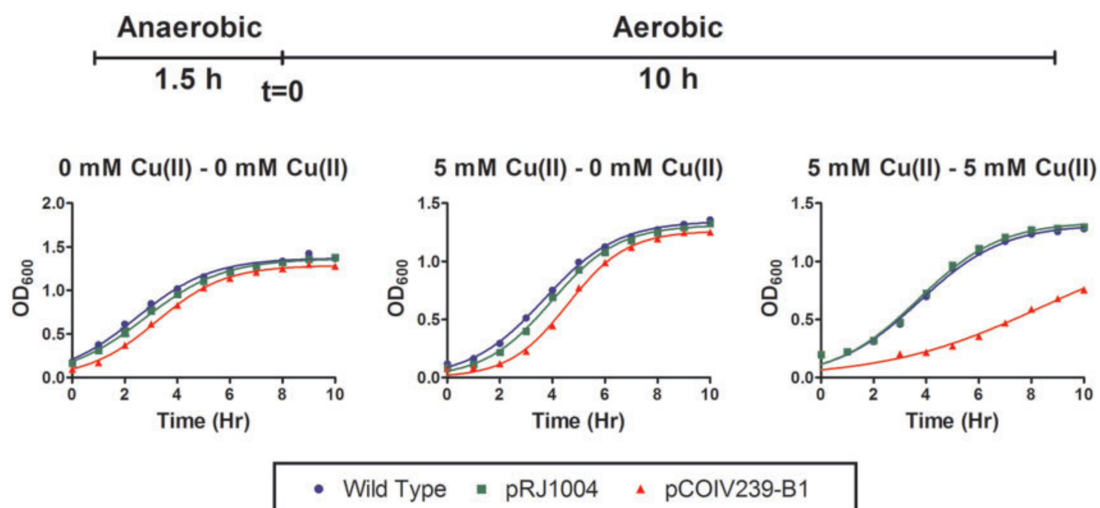


Fig. 3.—Copper tolerance of wild-type *Escherichia coli* BW25113 with resistance plasmids pRJ1004 and pCOIV239-B1. Growth curves mark 10-h aerobic growth after 90 min anaerobic incubation, with error bars indicating standard error of the mean for three replicates.

growth rate than wild-type cells or cells with pRJ1004 (P = 0.003 and P = 0.0013, respectively) when grown in LB media with no copper added (supplementary fig. S1, Supplementary Material online). W3110 cells with pCOIV239-B1 showed delayed growth relative to wild-type cells and cells with pRJ1004 (P = 0.0033 and P = 0.002, respectively) and also grew more slowly than the wild-type and pJR1004 cells (P = 0.0018 and P = 0.0434, respectively) when grown in LB media containing 2 mM $CuSO_4$ anaerobically then grown aerobically in LB with no copper added. W3110 cells were also grown anaerobically in LB media containing 2 mM $CuSO_4$ then switched to aerobic growth in LB with 2 mM $CuSO_4$. Under these conditions, cells with pRJ1004 had the highest growth rate, but this difference was only statistically significant in comparison with cells containing pCOIV239-B1 ($P < 0.0001$). Additionally, cells with pCOIV239-B1 showed a longer lag period when compared with wild-type cells and cells with pRJ1004 (P = 0.0333 and P = 0.0088, respectively). These trials revealed a statistical equivalence between wild-type cells and cells with pRJ1004 but a significant growth lag for cells with pCOIV239-B1 following a switch from anaerobic to aerobic growing conditions in both BW25113 and W3110 wild-type strains. These results suggest that the advantage of the complete CHASRI over a partial module in response to copper stress is most apparent in growth conditions where the availability of oxygen changes and show that the extent of the advantage can be dependent on the strain background. Combined with the results from the MTC experiments, these studies reveal a CHASRI phenotype that is robust to copper stress under a range of oxygen environments. However, the specific role of each component in copper and silver tolerance under different growth conditions remains to be resolved. In order to understand the evolutionary processes underpinning the formation these functions, we investigated the patterns of CHASRI gene diversification and synteny.

CHASRI Has a Spotty Distribution among Enterobacteria

We detected 1,099 related clusters (supplementary table S5, Supplementary Material online) of at least 2 homologs of CHASRI genes from a database of 1,150 complete bacterial genomes including their corresponding plasmids (supplementary table S2, Supplementary Material online). CHASRI has a sparse but taxonomically broad distribution (fig. 4) in the Enterobacteriaceae (gammaproteobacteria) and is found at least as often on plasmids as on chromosomes (figs. 5 and 6). However, the constituent *cus* and *pco* clusters are more likely to be located on chromosomes. The island was found in *Es. coli*, *En. cloacae*, *Klebsiella pneumoniae*, *Cronobacter turicensis*, *Cronobacter sakazakii*, and *Serratia marescens* strains, all but one strain of which (*Es. coli 8739* origin not known) are facultative anaerobic human or animal pathogens. CHASRIs detected in two *Shewanella* spp. isolated from the environment have a slightly different assemblage of genes, but were included as part of the overall distribution based on phylogenetic analysis results, which suggest that the original *pco* genes were replaced by an alternative set of genes including highly divergent homologs of *pcoA*, *pcoB*, *pcoC*, and *pcoD*. *Shewanella* islands also differ by containing a single two-component regulatory (*RS*) module, unlike other islands which contain *RS* modules associated with both the *cus* and the *pco* components. Transposon sequences usually directly flank (within two ORFs) both ends of CHASRIs (supplementary table S6, Supplementary Material online), with notable exceptions: 1) The *Shewanella* islands have three intervening sequences, one of which (YP_001181790) is annotated as a transposase that adjoins the expected recombination breakpoint; and 2) one of the two islands in *En. cloacae* ATCC 13407 is characterized by three transposon domains, which flank the *pco* cluster and also a complex of the *cus* cluster joined to a mercury resistance cluster on both sides.

The *cus* cluster, *cus-S-RCFBA* (genes transcribed in reverse indicated with "-"), is usually found independent of *pco* genes and restricted to the chromosomes of species in the Enterobacteriaceae (figs. 2 and 4). Independent *cus* clusters (*icus*) are fairly conserved within this lineage, with the notable exceptions of five *Shigella* spp. where it has experienced insertion, deletion, or complete loss, and also its absence from *Salmonella*, *Dickeya*, *Pantoea*, and *Erwinia*. A similar cluster, *czcBA.RS* (up to six intervening genes indicated with "."), involved in cobalt, zinc, and cadmium resistance (van der Lelie et al. 1997), is found in several lineages of betaproteobacteria and gammaproteobacteria, sometimes associated with homologs of *cusF* (a periplasmic metal-binding protein hypothesized to be involved in sequestration) (Xue et al. 2008) and/or *cus/czcC* (an outermembrane cation exporter) (Kim et al. 2010). In Enterobacteriaceae, canonical *pco* clusters (*pcoABCDRS*) are restricted to CHASRI, although *pcoABCD* is more widely distributed in the proteobacteria, often as *pco-S-RABCD*.

CHASRI Resulted from Sequential Combinations of Pre-existing Gene Modules

Phylogenetic analyses (fig. 5 and supplementary fig. S2A–S, Supplementary Material online) suggest that several collinearly transcribed constituent gene modules (*pcoAB*, *pcoCD*, *cusBA*, *cus/pcoRS*) were associated prior to the formation of the CHASRI and its constituent clusters. The most common association, *cusBA*, codes for an inner-membrane transporter and adapter (Rensing and Grass 2003) and is supported by these analyses to be homologous to *czcBA* modules. Homologs of *cus/czcC*, which complete the tripartite efflux complex as the outer membrane component for metal export (Su et al. 2011), are usually found upstream of *cusBA* homologs. *pcoABCD* contains a more widely distributed association, *pcoAB*, homologs of which are found in both beta and gammaproteobacteria. Evidence of alternative pairing of *pcoAB* homologs with

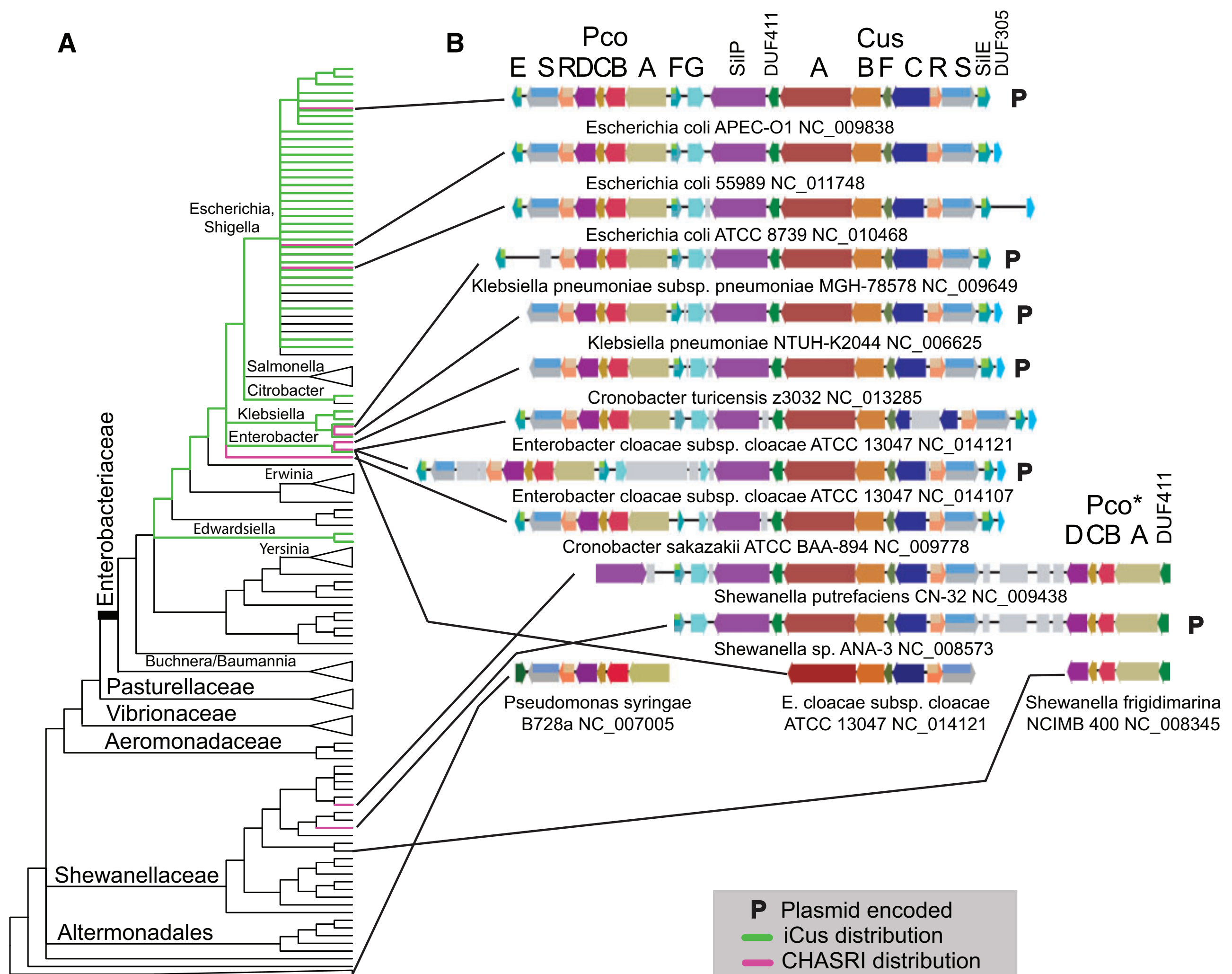


FIG. 4.—Distribution and structure of CHASRIs. (*A*) Distribution of CHASRI and i*cus* among proteobacteria. (*B*) To scale alignment of CHASRIs and recent ancestral modules detected in complete gammaproteobacteria proteomes. Genes with multiple colors are homologous to other genes in the cluster with any matching colors (i.e., pcoE, pcoF, and silE are homologous sequences).

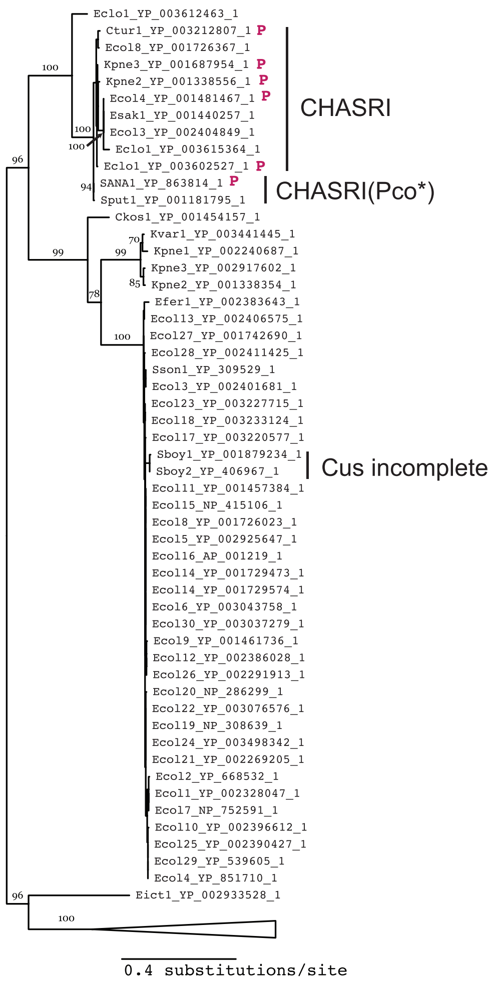
other genes can be found; in the *Acinetobacter baumannii* AB0057 genome (NC_011586) this module is adjacent to a *cus/czcC* homolog (YP_002318156.1), of the cation efflux family cl00316, which is involved in the export of Co, Zn, and Cd (van der Lelie et al. 1997). *pcoCD* function has not been well characterized, but its requirement in copper resistance has been shown to be variable between *cop* and *pco* homologs (Brown et al. 1995). Homologs of two-component sensor–regulator modules (*RS*) found in both *cus* and *pco* clusters are also found in multiple genomic contexts.

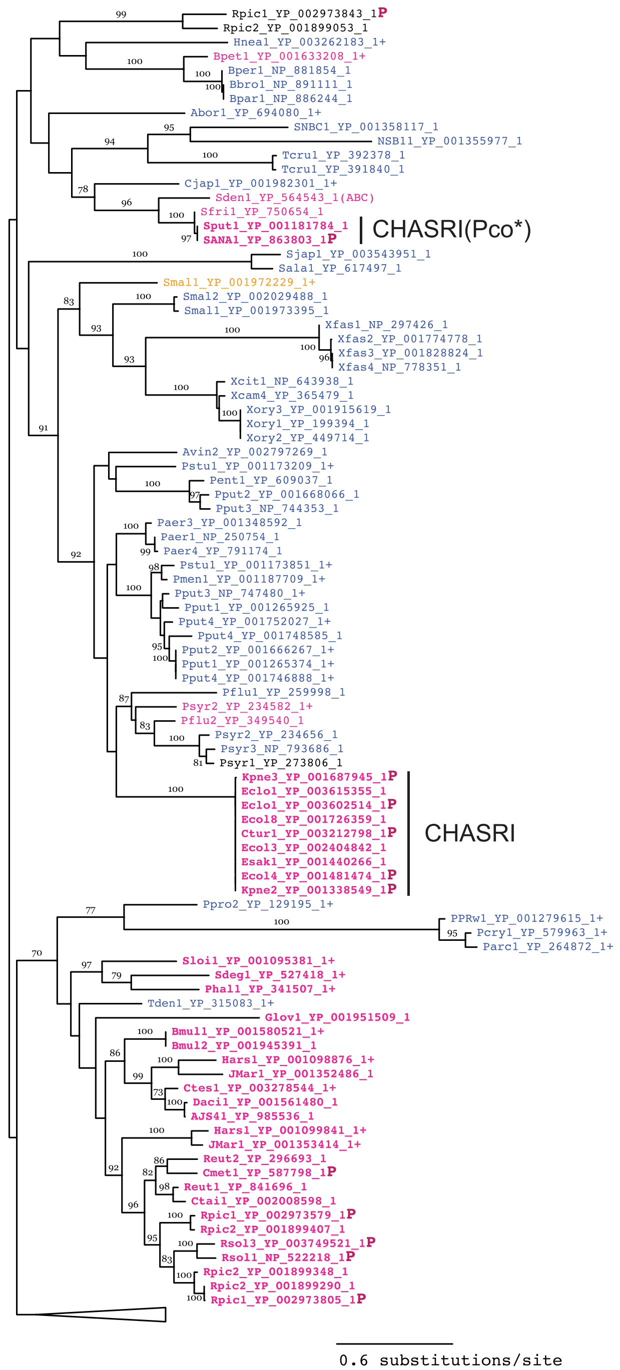
The distribution of putative module-associated genes shows bias toward presence in these modules and the modules show bias toward presence in clusters (supplementary table S5, Supplementary Material online). For example, 43 of 45 *pcoD* homologs detected are part of a precise *pcoCD* module and 32 homologs are part of a precise *pcoABCD* cluster. Similarly, out of 456 homologs of *cus/pcoS* detected, 410 homologs are part of precise *RS* modules, and out of 599 *cus/czcB* homologs detected, 454 homologs are part of precise *cus/czcBA* modules. Out of 101 *cusF* homologs detected, 79 homologs are clustered with *cus/czcBA* modules.

Although the modules inferred here are more often found on chromosomes than plasmids, plasmid occurrence rises with increased clustering. CHASRIs are enriched for plasmid occurrence (55% when including *Shewanella* islands) compared with all constituent modules.

CHASRI Evolution Is Distinct from Its Constituent Modules

Phylogenies of genes participating in the same putative module are highly correlated (fig. 5*A* and *B*). This is also supported by strong associations by shared reciprocal best BLAST hits between homologs of *cusA* and *cusB*, *pcoA* and *pcoB*,





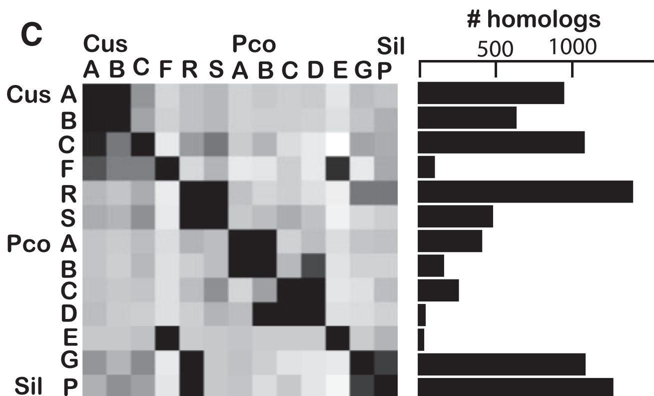


Fig. 5.—Diversity of CHASRI proteins. (*A*) ML amino acid phylogenies of CusB. (*B*) ML amino acid phylogenies of PcoB. Support values out of 100 ML bootstrap replicates are shown when ≥70. Color key displays clustering with functionally related genes. (*C*) The left panel is a heatmap of correlation of gene phylogenies expressed as the product of the proportion of shared reciprocal best BLAST hits for each gene, normalized by row. The right panel shows the number of homologs detected and analyzed for each gene.

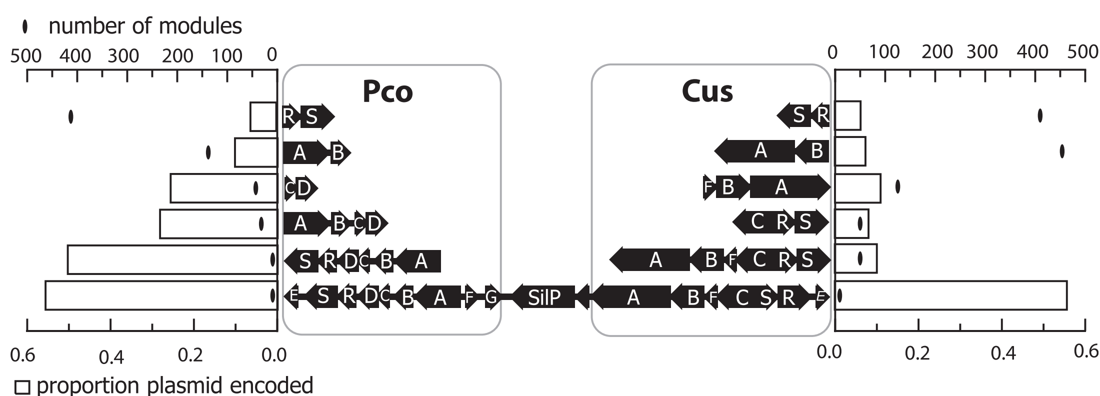


Fig. 6.—The association between plasmid encoding and aggregation of modules. Graph of the number of clusters shown in center panel of *pco* (left panel) and *cus* (right panel) genes. CHASRI clusters may not be of the exact synteny shown, but correspond to those in figure 4.

pcoC and *pcoD*, and *cus/pcoR* and *cus/pcoS* (fig. 5*C*). Associations are not as strong between pairs of genes belonging to alternative putative modules.

The adjacent taxon of CHASRI *cus* genes in gammaproteobacteria is usually the homolog from the chromosomally inherited *cus* cluster (i*cus*) in *En. cloacae* (fig. 5*A* and supplementary fig. S2*L–Q*, Supplementary Material online), generally with strong bootstrap support, although the position of the *En. cloacae* i*cus* sequence is sometimes within the CHASRI clan. *Enterobacter cloacae* i*cus* genes *cusR*, *cusS*, *cusC*, *cusF*, *cusB*, and *cusA* are monophyletic with CHASRI *cus* genes with 92%, 100%, 98%, 96%, 100%, and 100% ML bootstraps, respectively, excluding all other instances of i*cus*. i*cus* genes are more evenly distributed among Enterobacteria taxa and do not appear to conflict with the species phylogeny when CHASRI genes are excluded.

cus-R-SCFBA genes in clusters (i*cus* and CHASRI) split from all other assemblages of their constituent genes in ML trees, but genes in *pcoABCD* clusters appear in six clans in highly correlated *pcoA* and *pcoB* phylogenies among both betaproteobacteria and gammaproteobacteria (fig. 5 and supplementary fig. S2, Supplementary Material online) with varying levels of bootstrap support.

The homologs of genes directly participating in CHASRIs consistently form a well-supported clan, with the notable exception of two *Shewanella* spp. islands, in which the *cus* cluster, *pcoF*, *pcoG*, and *silP* are nested between an alternatively situated highly divergent *pcoABCD* cluster with an additional highly divergent *silP* homolog present in *S. putrifaciens* CN-32. ML phylogenies of combined nucleotide alignments illustrate that CHASRIs have experienced very little divergence (fig. 5 and supplementary fig. S2, Supplementary Material online), with at most 0.067 substitutions/site in GTR + gamma-optimized ML distance. The taxa represented within this small clan are diverse, including *Shewanella*, *Escherichia*, *Cronobacter*, *Enterobacter*, *Klebsiella*, and *Salmonella*, and within all but *Salmonella* both plasmid- and chromosome-encoded islands are found. *Shewanella* sequences formed a well-supported clan with *Es. coli* plasmid-encoded sequences (100% ML bootstraps). A notable, high-level sequence similarity exists within a clan of CHASRIs from animal and human pathogens, including *Salmonella* isolated from a cow in 2001 and *Cronobacter* from neonatal meningitis carried by a milk formula (Kucerova et al. 2010), also isolated in 2001.

Several other metal homeostasis genes are also constituents of the CHASRI. One gene family, containing *pcoE*, *pcoF*, and *silE*, was detected only in *Salmonella* spp. and CHASRIs (except for one divergent *silE* homolog adjacent to the i*cus* cluster in *Edwardsiella tarda*), where it appears to have undergone early diversification from a single original sequence. *pcoE*, a copper-binding periplasmic protein, exhibits the least sequence variation among taxa, followed by *pcoF* and *silE*. *pcoG* is a member of a large metalloendopeptidase family, for which over 1,000 sequences were recovered in the genome database. *pcoG* is often associated with *silP*, a putative copper-translocating P-type ATPase potentially cotranscribed with *cusCBFA*, for which over 1,100 sequences were recovered in the genome database.

A relaxed molecular clock (fig. 7*A*) was calibrated with a minimum date of 2,060 years at the divergence of *S. putrifaciens* strain CN32 and the closest related sequences from *Es. coli*. *Shewanella putrifaciens* strain CN32 was isolated (fig. 7*B*) from shale sandstone 230 m below ground level in the Morrison Formation in northwest New Mexico (Fredrickson et al. 1997) and contains a modified CHASRI that is phylogenetically nested in a clan of CHASRIs detected in farm animal and human-associated strains of *Es. coli*. Such a transfer of genetic material from the surface to deep subterranean rock microbiota would require an estimated 2,060 to 3,500 years given the local rate of ground water movement (Walvoord et al. 1999). The molecular clock estimates the beginning of CHASRI diversification at 5,477 years ago (4,659–16,370, 95% highest posterior density range [HPDR]), the divergence of *Shewanella* CHASRIs at 320 years ago (10–2,200 HPDR), and divergence of *pco*-associated *cus* from i*cus* in *En. cloacae* at 6,946 years ago (2,438–58,439 HPDR). These age estimates

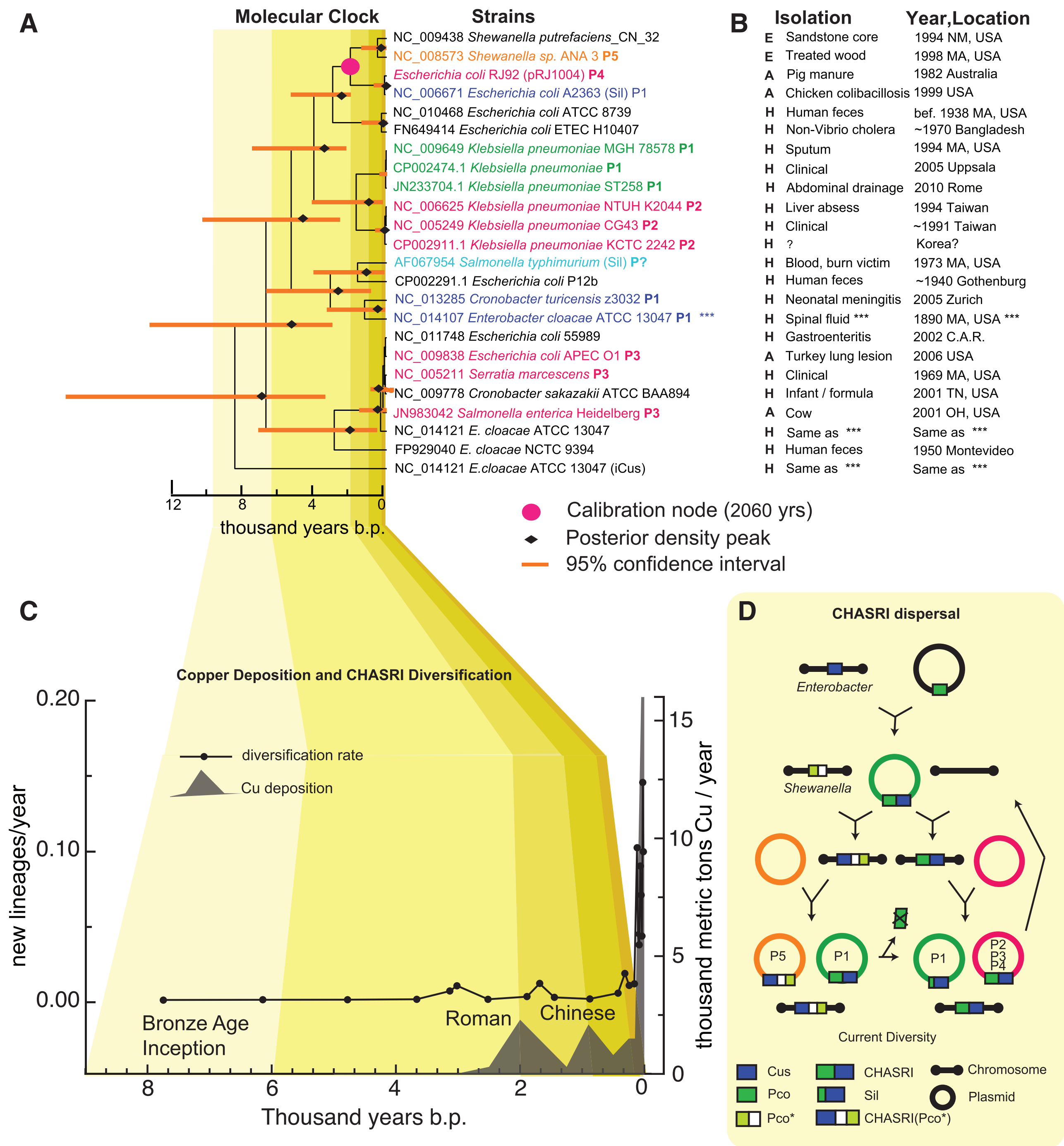


Fig. 7.—Origin and diversification of CHASRIs. (*A*) Chronogram generated in BEAST version 1.7.1 (Drummond and Rambaut 2007) from a CHASRI multigene nucleotide alignment. The aligned region includes *silE*, *cusSRCFBA*, and DUF411. Support values are out of 100 ML bootstraps. Taxon name coloring corresponds to plasmid synteny according to Mauve alignment (supplementary fig. S3, Supplementary Material online). Scale is calibrated with a 2,060-year minimum divergence between subterranean CHASRI and the most closely related surface CHASRI. (*B*) Description of CHASRI-containing strains in (*A*). Bold "E", "A," and "H" denote isolation in environment, agriculture, and hospital, respectively. Additional metal resistances are as reported in genome publications (parentheses indicate minimum resistance where complete genome was not available at the time of analyses.). (*C*) Graph of estimated global copper deposition due to human activity, reproduced from Hong et al. (1996). (*D*) Reconstruction of dispersal of CHASRIs, based on branching order in figure 5 and (*A*), and plasmid distributions shown in (*A*). A parsimonious reconstruction suggests that a chromosomal *cus* became linked with a plasmid *pco* in *Enterobacter*, and the resultant cluster was then distributed to new plasmids following insertion into chromosomes. Integration into a *Shewanella* chromosome resulted in recombination of CHASRI *cus* with native *Shewanella pco*, and *sil* clusters resulted from loss of *pco* from CHASRI.

are consistent with a total mutation rate of 2.5×10^{-8} mutations per site per generation (assuming 200 generations/year), which is significantly faster than the current estimate for nonsynonymous mutations in the greater *Es. coli* genome of 8.9×10^{-11}. Recently, the rate of sequence evolution was shown to scale with decreasing generation time in some bacteria (Weller and Wu 2015), and the observed mutation rate could also reflect horizontal transmission and selection (Wielgoss et al. 2011). A lineage through time plot of the median node heights from the BEAST analysis shows a general pattern of increased rate of CHASRI diversification coinciding with increased rates of copper deposition (Hong et al. 1996). Furthermore, three sharp accelerations and two intervening decelerations observed in CHASRI diversification could correspond to three major peaks of copper production (fig. 7*C*, Roman Empire, Chinese Sung Dynasty, and post-Industrial Revolution). Diversification among livestock-associated CHASRIs is reconstructed to be within the last century, while human-associated diversifications are both ancient and modern.

Discussion

A Copper/Silver Resistance Island Arose in a Close Relative of *Enterobacter cloacae* and Subsequently Experienced Dispersal by HGT

A copper homeostasis and silver resistance island (CHASRI), composed of *cus* and *pco* gene clusters and accessory genes situated between transposable elements, can more than triple copper resistance when conjugally transferred via plasmid to *Es. coli* that already possess chromosomal *cus* and *cue* copper resistance mechanisms (Tetaz and Luke 1983; Rouch et al. 1985). Analyses presented here suggest that the CHASRI formed by recombination between a chromosomally inherited *cus* cluster (i*cus*) in a close relative of *En. cloacae* and a *pco* cluster most closely related to sequences found on chromosomes in *Pseudomonas* spp. These analyses further supported rapid horizontal transfer of the CHASRI following assembly, which we detected by several methods (Zhaxybayeva and Doolittle 2011). Evidence for HGT dispersal of CHASRI is summarized as follows: 1) CHASRI as a cluster has a very spotty distribution compared with i*cus*, which is more commonly found on chromosomes of Enterobacteria; 2) CHASRI phylogenies conflict with corresponding species phylogenies, while i*cus* clusters are consistent; 3) CHASRI genes show very little divergence between different genera, compared with i*cus* clusters, suggesting that CHASRI diversification is more recent than generic divergences in Enterobacteria; 4) an analysis of complete bacterial genomes revealed that homologs of the modules that make up the CHASRI are primarily located on chromosomes, but plasmid-encoded CHASRIs outnumber chromosomal copies; and 5) CHASRIs, like other resistance islands, are often flanked by transposon sequences, which are suggestive of transferability (Paauw et al. 2010).

HGT of the CHASRI entails repeated exchange between plasmids and chromosomes, culminating in its presence on chromosomes in four genera and at least five distinct plasmid types. However, plasmids most similar to pRJ1004, the plasmid interrogated in this study, do not contain CHASRI. Conversely, plasmids with the most similar islands have no other regions of strong similarity with pRJ1004, suggesting CHASRI is not merely a passenger on a successful plasmid, but rather is itself a distinct object of selection. The silver-resistance cluster (*sil*) on *Salmonella typimurium* plasmid pMG101 (Silver 2003) and a convergently derived *sil*-like island on *Es. coli* A2363 plasmid pAPEC-O2-R (Johnson et al. 2005) arose after loss of *pco* genes from CHASRIs, which is supported by both phylogenetic analyses and the inclusion of *silP* and a member of the DUF411 gene family, which are otherwise only associated with *cus* clusters that are in CHASRIs.

Functional Integration of Interchangeable Modules Leads to Niche-Specific Functions

Analyses of CHASRIs in bacteria revealed several pairs of functionally coupled genes that are phylogenetically correlated and show strong conservation of synteny across a wide range of taxa. We consider these pairs to be functionally integrated modules retained by natural selection. These modules have been shown to have similar roles but different functions when found with different genomic neighbors. For example, the two-component regulatory systems CusRS and PcoRS share a substantial identity (60% for R, 37% for S) but they participate in regulation of different resistance pathways. Furthermore, two-component regulatory systems homologous to Cus/PcoRS in prokaryotes (Albright et al. 1989; Parkinson and Kofoid 1992; Mizuno 1997; Zhou et al. 2003) regulate a wide range of resistance genes and clustering of R and S is usually, but not always, ancestral (Koretke et al. 2000). This suggests that the functions of the two constituent genes are tightly linked, but the module itself is adaptable to diverse genomic and ecological roles. Similarly, resistance-nodulation-cell division (RND) efflux pumps (such as Cus/Czc) have been studied for their ability to detoxify a wide range of substrates. In addition to heavy metals, RND efflux pumps like the AcrAB-TolC system in *Es. coli* and MexAB-OprM system in *Pseudomonas aeruginosa* have been shown to export antibiotics including tetracycline and chloramphenicol, β-lactams, fusidic and nalidixic acid, fluoroquinolones, chemotherapeutics, sodium dodecyl sulfate (SDS), Triton X-100, and bile salts (Ma et al. 1995; Tsukagoshi and Aono 2000). In *P. aeruginosa*, a variety of outer membrane permeases (OMPs) have been shown to be interchangeable with certain AB modules (Murata et al. 2002). This functional interchangeability of modules is consistent with the reduced

phylogenetic correlation observed between OMPs and AB modules, suggesting this is an evolutionary mechanism for the emergence of novel efflux pathways.

Although the mechanistic roles of some of the *pco* constituents are unclear, PcoC and PcoD contribute to copper transport and ensure the full functionality of PcoABCD (Brown et al. 1995), similar to homologs CopC and CopD (Mellano and Cooksey 1988b; Cha and Cooksey 1993). PcoC is a small periplasmic protein that binds both Cu(I) and Cu(II) ions and interacts with MCO PcoA to oxidize Cu(I) to Cu(II) (Huffman et al. 2002; Wernimont et al. 2003), whereas the function of PcoD is still unknown. PcoCD homologs occur as the fusion protein YcnJ in *Bacillus subtilis*, further supporting the functional linkage of these genes (Chillappagari et al. 2009). The combination of mechanisms that contribute to the spatial clustering of functionally linked genes has been discussed widely (Lawrence and Roth 1996; Hurst et al. 2002; Baquero 2004); our results are consistent with horizontal transfer, linkage, and coexpression as drivers of clustering.

CHASRI Is a "Winning Pattern" Module Combination that is Successful Under Human-Induced Copper Pressure

CHASRI is the most complex aggregation of copper resistance modules known and is highly prone to dispersal by horizontal transfer. The potential for complementation in this aggregation of gene modules may be directly linked to CHASRI success. For example, coordination of periplasmic and extracellular copper transport could explain the increased resistance conferred by CopABCD combined modules over CopAB modules (Mellano and Cooksey 1988a). The *cue* system, which detoxifies copper with an MCO in the presence of oxygen (Outten et al. 2000; Grass and Rensing 2001), co-occurs with i*cus* systems for efflux under aerobic and anaerobic conditions (Outten et al. 2001) in facultative anaerobes. CHASRIs combine MCO copper detoxification by *pco* with efflux by *cus* in a single island. We have shown that the CHASRI on pRJ1004 enhances aerobic copper resistance over wild-type BW25113 cells but provides less resistance than a plasmid that contains only *pco*. However, cells with the *pco*-only construct are severely inhibited upon transition from anaerobic to aerobic copper exposure, while CHASRI-containing cells tolerate these transitions as well as control cells containing i*cus* and *cue* only. This suggests that the combined gene cluster confers a more environmentally robust resistance to copper than the components, *cus* and *pco*, in isolation. This type of resistance is likely to provide the greatest benefit to facultative anaerobes that natively lack both *cus* and *pco* systems. We suggest that CHASRI is a so-called "winning pattern," which arose in a copper-stressed environment that favored a range of forms of copper resistance and drove module combination (fig. 7*D*) through genetic linkage (Baquero 2004). Since its assembly, this resistance island has become highly selectable in copper-rich sites such as mines, agriculture, hospitals, and sewage. All recent diversity of CHASRI appears to involve horizontal transfers, and the rate of HGT increases with integration of copper-resistance modules. This is consistent with HGT-elevated fitness of genes that are found in functionally integrated gene clusters (Lawrence and Roth 1996).

The Origin and Diversification of CHASRI Are associated with Long-Term Trends of Human Copper Emissions

The horizontal transfer of a CHASRI from Enterobacteria to *Shewanella* provided an opportunity to model CHASRI origin and diversification through time using a molecular clock, assuming a minimum surface–subterranean separation date. By applying the 2,060-year calibration to the *Es. coli/Shewanella* divergence in a relaxed molecular clock analysis of CHASRI nucleotides, we were able to associate CHASRI origin and episodes of accelerated diversification with multimillennial trends in anthropogenic copper emissions. We inferred that the current CHASRI diversity originated in a relative of *En. cloacae* around 6,000 years ago, which corresponds to current estimates of the beginning of the Bronze Age (Radivojevic et al. 2010), when anthropogenic copper became a factor in environmental stress. The high level of resistance (20 mM) conferred by the CHASRI (Tetaz and Luke 1983) compared with the low level of copper (1.6×10^{-5} mM) in uncontaminated fresh water (Alberta Environmental Protection 1997) suggests that CHASRI most likely diversified under broad copper stress driven by human metallurgical activity. We speculate that the likely donors of *cus* (*En. cloacae*) and *pco* (*P. fluorescens*) could have been cocontaminants of dairy fermented in copper alloy vessels by Bronze Age agrarians (Canganella et al. 1999). Alternatively, these resistance mechanisms became clustered under elevated environmental copper conditions and were later acquired from standing diversity by bacteria in animal microbiomes (Sander and Koschinsky 2011).

Greenland ice cores support peaks in atmospheric copper emissions during the time of the Roman Empire 2,000 years ago, again 1,100 years ago in China, and most notably in the last 150 years (Hong et al. 1996). In our model, estimates of copper emissions correspond in number and magnitude to episodic accelerations in CHASRI diversification. The origin of CHASRI in deep subterranean *Shewanella* in the Southwestern United States may be linked to the pre-Christian Era copper industry (Ehrhardt 2009). Under this model the estimated divergence between surface and subterranean *Shewanella* CHASRIs corresponds to colonial mining in the American Southwest approximately 300 years ago, which suggests that the modified form of CHASRI was reintroduced to the surface. This model is further supported by two *K. pneumoniae* CHASRIs, found on nearly identical plasmids 3 years apart, which are estimated to have diverged for less than 10 years. An alternative model that places the subterranean divide at the divergence of *Shewanella* islands does not

match the above dates directly but still suggests recent diversification following an origin ~9,000 years ago (supplementary table S7, Supplementary Material online). Although we find the visual appearance of cointegration of the CHASRI diversification and ancient anthropogenic copper deposition rate curves to be suggestive of an association, we caution that specific dates inferred by molecular clock analyses are dependent on multiple assumptions including reliable calibration points and the generation times of the bacteria in question, which cannot now be estimated with high certainty.

The current known distribution of CHASRIs is likely driven by tremendous copper pressure exerted by human activities in the 19th and 20th centuries. In the late 19th century, in addition to environmental emissions from smelting, copper was widely used in food preservation and as a disinfectant of sewage, gastroenteritis stools, and wounds (Blyth 1888; Rideal 1895; Young 1898). Since 1955, copper has been used for growth stimulation in large-scale swine and poultry production (Barber et al. 1955), the mechanism of which may involve selection on livestock gut microbiome communities (Shurson et al. 1990; Dunning et al. 1998). Furthermore, elevated copper in tap water due to the corrosion of copper plumbing may give a selective advantage to intestine-associated bacteria with copper-resistance mechanisms. The earliest isolated CHASRIs date to 1890, in *En. cloacae* strain ATCC13047 isolated from the spinal fluid of a patient with meningitis. Genome analysis revealed that this single strain contains representatives of two major clans of CHASRIs. Highly similar CHASRIs are found in the genomes of pathogenic Enterobacteria on five continents, *Es. coli* poultry respiratory infections, *Cronobacter* responsible for neonatal death, gastroenteritic *Es. coli*, and *Klebsiella* responsible for pneumonia and meningitis (Munson et al. 2000; Silver 2003; Chen et al. 2004; Gilmour et al. 2004; Johnson et al. 2005; Houdt et al. 2009; Crossman et al. 2010; Su et al. 2011).

An Evolutionary Prospective for Emergent Pathogens

The rapid and widespread dispersal of copper resistance islands illustrates a dramatic flux of bacterial genes by horizontal transfer in response to human influence (Bezuidt et al. 2011). Most bacterial strains in which CHASRIs were detected also show resistance to silver, arsenic, and tellurium, suggesting the island could be associated with multiple selective pressures involving metal toxicity. Human-driven mobilization of silver has been more limited than copper, but medicinal applications of antimicrobial silver (Edwards-Jones 2009) may have contributed to the spread of CHASRI (McHugh et al. 1975). Previous research has linked increased pathogen durability (Walther and Ewald 2004), specifically pathogen metal resistance (Audic et al. 2007; Kucerova et al. 2010; Shafeeq et al. 2011), with increased virulence, suggesting that CHASRI-encoded resistance is part of a survivability mechanism that can be acquired by strains in animal microbiomes transitioning from commensal to pathogenic organisms (Duriez et al. 2001).

Recently, it has been shown that copper is involved in macrophage defense against *Salmonella* infections (Osman et al. 2010; Achard et al. 2012), highlighting a mechanism for CHASRIs to provide an additional fitness benefit to pathogenic organisms. Because DNA-based resistance islands are not continuously bound to living cells, or even to specific plasmid vectors over the long term, control of resistance dispersal must involve improving upstream management practices to prevent selection for such mechanisms in sympatric bacteria, rather than downstream antibacterial measures, which are likely to exacerbate disease emergence. Together, these results suggest that an ecological disturbance, driven by human activity starting in the Bronze Age, has influenced the contents of gammaproteobacterial pan-genomes, affected bacterial resistance in far-flung environments, and perhaps contributed to the emergence of some modern pathogens.

Supplementary Material

Supplementary tables S1–S7 and figures S1–S3 are available at *Genome Biology and Evolution online* (http://www.gbe.oxfordjournals.org/).

Acknowledgments

Sanger sequencing was performed by the Genomics Core at Northwestern University. Illumina genome sequencing was performed by the Vanderbilt University Genome Technology Core. This work was supported in part by NIH Grant R01GM038784 (T.V.O.).

Note Added in Proof

Recent reviews on related copper resistance (Hao et al. 2015) and silver resistance islands have been published (Randall et al. 2015).

Literature Cited

Achard MES, et al. 2012. Copper redistribution in murine macrophages in response to *Salmonella* infection. Biochem J. 444:51–57.

Alberta Environment & Sustainable Resource Development (ESRD). 2014. Environmental Quality Guidelines for Alberta Surface Waters. Water Policy Branch, Policy Division. Edmonton. p. 48.

Albright LM, Huala E, Ausubel FM. 1989. Prokaryotic signal transduction mediated by sensor and regulator protein pairs. Annu Rev Genet. 23:311–336.

Altschul SF, et al. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

Audic S, et al. 2007. Genome analysis of *Minibacterium massiliensis* highlights the convergent evolution of water-living bacteria. PLoS Genet. 3:e138

Baquero F. 2004. From pieces to patterns: evolutionary engineering in bacterial pathogens. Nat Rev Microbiol. 2:510–518.

Barber R, Braude R, Mitchell K, Cassidy J. 1955. High copper mineral mixture for fattening pigs. Chem Ind. 21:601–603.

Bezuidt O, Pierneef R, Mncube K, Lima-Mendez G, Reva ON. 2011. Mainstreams of horizontal gene exchange in enterobacteria: consideration of the outbreak of enterohemorrhagic *E. coli* O104:H4 in Germany in 2011. PLoS One 6:e25702.

Blyth AW. 1888. Foods: their composition and analysis. London: Charles Griffin and Company.

Brown NL, Barrett SR, Camakaris J, Lee BT, Rouch DA. 1995. Molecular genetics and transport analysis of the copper-resistance determinant (pco) from *Escherichia coli* plasmid pRJ1004. Mol Microbiol. 17:1153–1166.

Bryson JW, et al. 1993. Chemical and genetic studies of copper resistance in *E. coli*. In: Karlin KD, Tyler Z, editors. Bioinorganic chemistry of copper. New York: Chapman & Hall. p. 101–109.

Campbell MA, Rokas A, Slot JC. 2012. Horizontal transfer and death of a fungal secondary metabolic gene cluster. Genome Biol Evol. 4:289–293.

Canganella F, Nespica M, Giontella D, Trovatelli L. 1999. Survival of *Enterobacter cloacae* and *Pseudomonas paucimobilis* in yoghurts manufactured from cow's milk and soymilk during storage at different temperatures. Microbiol Res. 154:15–21.

Cha JS, Cooksey DA. 1993. Copper hypersensitivity and uptake in *Pseudomonas syringae* containing cloned components of the copper resistance operon. Appl Environ Microbiol. 59:1671–1674.

Chen YT, et al. 2004. Sequencing and analysis of the large virulence plasmid pLVPK of *Klebsiella pneumoniae* CG43. Gene 337:189–198.

Chillappagari S, Miethke M, Trip H, Kuipers OP, Marahiel MA. 2009. Copper acquisition is mediated by YcnJ and regulated by YcnK and CsoR in *Bacillus subtilis*. J Bacteriol. 191:2362–2370.

Cohen SN, Chang ACY. 1977. Revised interpretation of the origin of the pSC101 plasmid. J Bacteriol. 132:734–737.

Cooksey DA. 1987. Characterization of a copper resistance plasmid conserved in copper-resistant strains of *Pseudomonas syringae* pv. *tomato*. Appl Environ Microbiol. 53:454–456.

Crossman LC, et al. 2010. A commensal gone bad: complete genome sequence of the prototypical enterotoxigenic *Escherichia coli* strain H10407. J Bacteriol. 192:5822–5831.

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol. 7:214.

Dunning JC, Ma Y, Marquis RE. 1998. Anaerobic killing of oral streptococci by reduced, transition metal cations. Appl Environ Microbiol. 64:27–33.

Duriez P, et al. 2001. Commensal *Escherichia coli* isolates are phylogenetically distributed among geographically distinct human populations. Microbiology 147:1671–1676.

Edwards-Jones V. 2009. The benefits of silver in hygiene, personal care and healthcare. Lett Appl Microbiol. 49:147–152.

Ehrhardt KL. 2009. Copper Working Technologies, contexts of use, and social complexity in the Eastern Woodlands of Native North America. J World Prehist. 22:213–235.

Finney LA, O'Halloran TV. 2003. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science 300:931–936.

Franke S, Grass G, Rensing C, Nies DH. 2003. Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J Bacteriol. 185:3804–3812.

Fredrickson J, et al. 1997. Pore-size constraints on the activity and survival of subsurface bacteria in a late cretaceous shale-sandstone sequence, northwestern New Mexico. Geomicrobiol J. 14:183–202.

Fung DKC, Lau WY, Chan WT, Yan A. 2013. Copper efflux is induced during anaerobic amino acid limitation in *Escherichia coli* to protect iron-sulfur cluster enzymes and biogenesis. J Bacteriol. 195:4556–4568.

Gibbons JG, et al. 2009. Benchmarking next-generation transcriptome sequencing for functional and evolutionary genomics. Mol Biol Evol. 26:2731–2744.

Gilmour MW, Thomson NR, Sanders M, Parkhill J, Taylor DE. 2004. The complete nucleotide sequence of the resistance plasmid R478: defining the backbone components of incompatibility group H conjugative plasmids through comparative genomics. Plasmid 52:182–202.

Grass G, Rensing C. 2001. CueO is a multi-copper oxidase that confers copper tolerance in *Escherichia coli*. Biochem Biophys Res Commun. 286:902–908.

Gupta A, Matsui K, Lo JF, Silver S. 1999. Molecular basis for resistance to silver cations in *Salmonella*. Nat Med. 5:183–188.

Hao, et al. 2015. Survival in amoeba-a major selection pressure on the presence of bacterial copper and zinc resistance determinants? Identification of a 'Copper Pathogenicity Island'. Appl Microbiol Biotechnol. 99:5817–5824.

Hobman J, Crossman L. 2015. Bacterial antimicrobial metal ion resistance. J Med Microbiol. 64:471–497.

Hong S, Candelone J, Patterson C, Boutron C. 1996. History of ancient copper smelting pollution during Roman and medieval times recorded in Greenland ice. Science 272:246–249.

Houdt R, Monchy S, Leys N, Mergeay M. 2009. New mobile genetic elements in *Cupriavidus metallidurans* CH34, their possible roles and occurrence in other bacteria. Antonie Van Leeuwenhoek 96:205–226.

Huang SH, et al. 1999. Identification and characterization of an *Escherichia coli* invasion gene locus, ibeB, required for penetration of brain microvascular endothelial cells. Infect Immun. 67:2103–2109.

Huffman DL, et al. 2002. Spectroscopy of Cu(II)-PcoC and the multicopper oxidase function of PcoA, two essential components of *Escherichia coli* pco copper resistance operon. Biochemistry 41:10046–10055.

Hurst L, Williams E, Pál C. 2002. Natural selection promotes the conservation of linkage of co-expressed genes. Trends Genet. 18:604–606.

Jensen KF. 1993. The *Escherichia coli* K-12 "wild types" W3110 and MG1655 have an rph frameshift mutation that leads to pyrimidine starvation due to low pyrE expression levels. J Bacteriol. 175:3401–3407.

Johnson TJ, Siek KE, Johnson SJ, Nolan LK. 2005. DNA sequence and comparative genomics of pAPEC-O2-R, an avian pathogenic *Escherichia coli* transmissible R plasmid. Antimicrob Agents Chemother. 49:4681–4688.

Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30:3059–3066.

Katoh K, Toh H. 2008. Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform. 9:286–298.

Kim EH, Rensing C, Mcevoy MM. 2010. Chaperone-mediated copper handling in the periplasm. Nat Prod Rep. 27:711–719.

Koretke KK, Lupas AN, Warren PV, Rosenberg M, Brown JR. 2000. Evolution of two-component signal transduction. Mol Biol Evol. 17:1956–1970.

Kucerova E, et al. 2010. Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridization analysis with other *Cronobacter* species. PLoS One 5:e9556

Lawrence J, Roth J. 1996. Selfish operons: horizontal transfer may drive the evolution of gene clusters. Genetics 143:1843–1860.

Lee YA, Hendson M, Panopoulos NJ, Schroth MN. 1994. Molecular cloning, chromosomal mapping, and sequence analysis of copper resistance genes from *Xanthomonas campestris* pv. *juglandis*: homology with small blue copper proteins and multicopper oxidase. J Bacteriol. 176:173–188.

Li L. 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. 13:2178–2189.

Ma D, et al. 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol Microbiol. 16:45–55.

Magyar JS, Godwin HA. 2003. Spectropotentiometric analysis of metal binding to structural zinc-binding sites: accounting quantitatively for pH and metal ion buffering effects. Analytical Biochem. 320:39–54.

McHugh GL, Moellering RC, Hopkins CC, Swartz MN. 1975. *Salmonella typhimurium* resistant to silver nitrate, chloramphenicol, and ampicillin. Lancet 1:235–240.

Mellano MA, Cooksey DA. 1988a. Induction of the copper resistance operon from *Pseudomonas syringae*. J Bacteriol. 170:4399–4401.

Mellano MA, Cooksey DA. 1988b. Nucleotide sequence and organization of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. J Bacteriol. 170:2879–2883.

Mizuno T. 1997. Compilation of all genes encoding two-component phosphotransfer signal transducers in the genome of *Escherichia coli*. DNA Res. 4:161–168.

Munson GP. 1997. Copper resistance of enteric bacteria: molecular and genetic analysis of resistance, homeostasis, and transcriptional regulation [phd thesis]. [Evanston (IL)]: Northwestern University.

Munson GP, Lam DL, Outten FW, O'Halloran TV. 2000. Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. J Bacteriol. 182:5864–5871.

Murata T, Kuwagaki M, Shin T, Gotoh N, Nishino T. 2002. The substrate specificity of tripartite efflux systems of *Pseudomonas aeruginosa* is determined by the RND component. Biochem Biophys Res Commun. 299:247–251.

Nies DH. 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev. 27:313–339.

Osman D, et al. 2010. Copper homeostasis in *Salmonella* is atypical and copper-CueP is a major periplasmic metal complex. J Biol Chem. 285:25259–25268.

Outten FW, Huffman DL, Hale JA, O'Halloran TV. 2001. The independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. J Biol Chem. 276:30670–30677.

Outten FW, Outten CE, Hale J, O'Halloran TV. 2000. Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal MerR homologue, cueR. J Biol Chem. 275:31024–31029.

Paauw A, Leverstein-van Hall MA, Verhoef J, Fluit AC. 2010. Evolution in quantum leaps: multiple combinatorial transfers of HPI and other genetic modules in Enterobacteriaceae. PLoS One 5:e8662.

Parkinson JS, Kofoid EC. 1992. Communication modules in bacterial signaling proteins. Annu Rev Genet. 26:71–112.

Pontel LB, Soncini FC. 2009. Alternative periplasmic copper-resistance mechanisms in Gram negative bacteria. Mol Microbiol. 73:212–225.

Radivojevic M, et al. 2010. On the origins of extractive metallurgy: new evidence from Europe. J Archaeol Sci. 37:2775–2787.

Randall, et al. 2015. Silver resistance in Gram-negative bacteria: a dissection of endogenous and exogenous mechanisms. J Antimicrobial Chem. 70:1037–1046.

Rensing C, Fan B, Sharma R, Mitra B, Rosen BP. 2000. CopA: an Escherichia Coli Cu(I)-Translocating P-Type ATPase. Proc Natl Acad Sci U S A. 97(2):652–656.

Rensing C, Grass G. 2003. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol Rev. 27:197–213.

Rideal S. 1895. Disinfection and disinfectants. London: Charles Griffin and Company.

Rouch D, Camakaris J, Lee BT, Luke RK. 1985. Inducible plasmid-mediated copper resistance in *Escherichia coli*. J Gen Microbiol. 131:939–943.

Rouch DA, Brown NL. 1997. Copper-inducible transcriptional regulation at two promoters in the *Escherichia coli* copper resistance determinant pco. Microbiology 143:1191–1202.

Sander SG, Koschinsky A. 2011. Metal flux from hydrothermal vents increased by organic complexation. Nat Geosci. 4:145–150.

Shafeeq S, et al. 2011. The cop operon is required for copper homeostasis and contributes to virulence in *Streptococcus pneumoniae*. Mol Microbiol. 81:1255–1270.

Shurson GC, Ku PK, Waxler GL, Yokoyama MT, Miller ER. 1990. Physiological relationships between microbiological status and dietary copper levels in the pig. J Anim Sci. 68:1061–1071.

Silver S. 2003. Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. FEMS Microbiol Rev. 27:341–353.

Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690.

Stoyanov JV, Hobman JL, Brown NL. 2001. CueR (YbbI) of Escherichia coli is a MerR Family Regulator Controlling Expression of the Copper Exporter CopA. Proc Mol Microbiol. 39:502–511.

Su CC, Long F, Yu EW. 2011. The Cus efflux system removes toxic ions via a methionine shuttle. Protein Sci. 20:6–18.

Swofford DL. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sunderland (MA): Sinauer Associates.

Tetaz TJ, Luke RK. 1983. Plasmid-controlled resistance to copper in *Escherichia coli*. J Bacteriol. 154:1263–1268.

Tsukagoshi N, Aono R. 2000. Entry into and release of solvents by *Escherichia coli* in an organic-aqueous two-liquid-phase system and substrate specificity of the AcrAB-TolC solvent-extruding pump. J Bacteriol. 182:4803–4810.

van der Lelie D, et al. 1997. Two-component regulatory system involved in transcriptional control of heavy-metal homoeostasis in *Alcaligenes eutrophus*. Mol Microbiol. 23:493–503.

Vandermeer J. 2010. How populations grow: the exponential and logistic equations. Nat Educ Knowledge. 3:15.

Voloudakis AE, Bender CL, Cooksey DA. 1993. Similarity between copper resistance genes from *Xanthomonas campestris* and *Pseudomonas syringae*. Appl Environ Microbiol. 59:1627–1634.

Walther BA, Ewald PW. 2004. Pathogen survival in the external environment and the evolution of virulence. Biol Rev Camb Philos Soc. 79:849–869.

Walvoord MA, et al. 1999. Groundwater flow and geochemistry in the Southeastern San Juan Basin: implications for microbial transport and activity. Water Resour Res. 35:1409–1424.

Weller C, Wu M. 2015. A generation-time effect on the rate of molecular evolution in bacteria. Evolution 69:643–652.

Wernimont AK, et al. 2003. Crystal structure and dimerization equilibria of PcoC, a methionine-rich copper resistance protein from *Escherichia coli*. J Biol Inorg Chem. 8:185–194.

Wielgoss S, et al. 2011. Mutation rate inferred from synonymous substitutions in a long-term evolution experiment with *Escherichia coli*. G3 (Bethesda) 1:183–186.

Xue Y, et al. 2008. Cu(I) recognition via cation-pi and methionine interactions in CusF. Nat Chem Biol. 4:107–109.

Young AG. 1898. Notes on disinfectants and disinfection. Augusta (GA): Kennebec Journal Print.

Zhaxybayeva O, Doolittle WF. 2011. Lateral gene transfer. Curr Biol. 21:R242–R246.

Zhou L, Lei XH, Bochner BR, Wanner BL. 2003. Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. J Bacteriol. 185:4956–4972.

Associate editor: Eric Bapteste