creased, and body mass was lower when squirrels were feeding on these items as compared with feeding on seed (*26*, *27*). Lastly, food supplementation experiments of American reds have failed to produce increases in litter size or a second litter, providing strong evidence that the increased reproductive investment observed in our study cannot be triggered by increased energy availability alone (*17*, *28*, *29*). We hypothesize that, rather than following a resource-tracking strategy where reproductive investment is determined by current resource amounts, reproductive rates are driven by future fitness payoffs. During years of low seed production, competition among juveniles for available resources is intense, and, although litter augmentation experiments in American reds show that females are capable of supporting larger litters (*30*), they refrain from doing so because offspring recruitment is low (*30*). However, when mast years occur, competition among juveniles is reduced, and females produce more offspring, which successfully recruit into the population (*31*, *32*). Further, the increased production of young by females in mast years does not come with any obvious cost to the female, because overwinter survival is not reduced after years of increased reproductive investment (for American reds, offspring production in the previous year versus proportion of adult females surviving to spring has slope = –0.023 ± 0.034, $t_{15} = -0.7$, and $P = 0.51$; for Eurasian reds, proportion of estrous females in the previous year versus adult female survival to spring has slope = 0.37 ± 0.27, $t_{15} = 1.4$, and $P = 0.19$).

If masting has evolved as a swamp-and-starve adaptation against seed predation, then anticipatory reproduction and population growth represent a potent counteradaptation by the predators. Given that increased reproductive output in these systems coincides with low current but high future resources, our results suggest that reproductive investment in these systems is more responsive to future fitness prospects than present energetic constraints. The evolution of seed masting in trees is also driven by the survival prospects for progeny rather than simple resource tracking, suggesting an intriguing parallel in reproductive strategies of trees and the predators that consume their seed.

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Human Catechol-*O*-Methyltransferase Haplotypes Modulate Protein Expression by Altering mRNA Secondary Structure

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Catechol-*O*-methyltransferase (COMT) is a key regulator of pain perception, cognitive function, and affective mood. Three common haplotypes of the human *COMT* gene, divergent in two synonymous and one nonsynonymous position, code for differences in COMT enzymatic activity and are associated with pain sensitivity. Haplotypes divergent in synonymous changes exhibited the largest difference in COMT enzymatic activity, due to a reduced amount of translated protein. The major *COMT* haplotypes varied with respect to messenger RNA local stem-loop structures, such that the most stable structure was associated with the lowest protein levels and enzymatic activity. Site-directed mutagenesis that eliminated the stable structure restored the amount of translated protein. These data highlight the functional significance of synonymous variations and suggest the importance of haplotypes over single-nucleotide polymorphisms for analysis of genetic variations.

The ability to predict the downstream effects of genetic variation is critically important for understanding both the evolution of the genome and the molecular basis of human disease. The effects of nonsynonymous polymorphisms have been widely characterized; because these variations directly influence protein function, they are relatively easy to study statistically and experimentally (*1*). However, characterizing polymorphisms located in regulatory regions, which are much more common, has proved to be problematic (*2*). Here, we focus on the mechanism whereby polymorphisms of the cathechol-*O*-methyltransferase (*COMT*) gene regulate gene expression.

COMT is an enzyme responsible for degrading catecholamines and thus represents a critical component of homeostasis maintenance (*3*). The human *COMT* gene encodes two distinct proteins: soluble COMT (S-COMT) and membrane-bound COMT (MB-COMT) through the use of alternative translation initia-

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tion sites and promoters (*3*). Recently, COMT has been implicated in the modulation of persistent pain (*4*–*7*). Our group demonstrated that three common haplotypes of the human *COMT* gene are associated with pain sensitivity and the likelihood of developing temporomandibular joint disorder (TMJD), a common chronic musculoskeletal pain condition (*4*). Three major haplotypes are formed by four single-nucleotide polymorphisms (SNPs): one located in the *S-COMT* promoter region (A/G; rs6269) and three in the *S-* and *MB-COMT* coding region at codons $his^{62}his$ (C/T; rs4633), $leu^{136}leu$ (C/G; rs4818), and $val^{158}met$ (A/G; rs4680) (Fig. 1A). On the basis of subjects' pain responsiveness, haplotypes were designated as low (LPS; GCGG), average (APS; ATCA), or high (HPS; ACCG) pain sensitive. Individuals carrying HPS/APS or APS/APS diplotypes were nearly 2.5 times as likely to develop TMJD. Previous data further suggest that *COMT* haplotypes code for differences in COMT enzymatic activity (*4*); however, the molecular mechanisms involved have remained unknown.

A common SNP in codon 158 ($val^{158}met$) has been associated with pain ratings and μ-opioid system responses (*7*) as well as addiction, cognition, and common affective disorders (*3*, *8*–*10*). The substitution of valine (Val) by methionine (Met) results in reduced thermostability and activity of the enzyme (*3*). It is generally accepted that $val^{158}met$ is the main source of individual variation in human COMT activity; numerous studies have identified associations between the low-activity *met* allele and several complex phenotypes (*3*, *8*, *10*). However, observed associations between these conditions and the *met* allele are often modest and occasionally inconsistent (*3*). This suggests that additional SNPs in the *COMT* gene modulate COMT activity. Indeed, we found that haplotype rather than an individual SNP better accounts for variability in pain sensitivity (*4*). The HPS and LPS haplotypes that both code for the stable val^{158} variant were associated with the two extreme-pain phenotypes; thus, the effect of haplotype on pain sensitivity in our study cannot be explained by the sum of the effects of functional SNPs. Instead, the $val^{158}met$ SNP interacts with other SNPs to determine phenotype. Because variation in the S-COMT promoter region does not contribute to pain phenotype (Fig. 1A), we suggest that the rate of mRNA degradation or protein synthesis is affected by the structural properties of the haplotypes, such as haplotype-specific mRNA secondary structure.

Previous reports have shown that polymorphic alleles can markedly affect mRNA secondary structure (*11*, *12*), which can then have functional consequences on the rate of mRNA degradation (*11*, *13*). It is also plausible that polymorphic alleles directly modulate protein translation through alterations in mRNA secondary structure, because protein translation efficiency is affected by mRNA secondary structure (*14*–*16*). To test these possibilities, we evaluated the affect of LPS, APS, and HPS haplotypes on the stability of the corresponding mRNA secondary structures (*17*).

Secondary structures of the full-length LPS, APS, and HPS mRNA transcripts were predicted by means of the RNA Mfold (*18*, *19*) and Afold (*20*) programs. The mRNA folding analyses demonstrated that the major *COMT* haplotypes differ with respect to mRNA secondary structure. The LPS haplotype codes for the shortest, least stable local stem-loop structure, and the HPS haplotype codes for the longest, most stable local stem-loop structure in the val^{158} region for both *S-COMT* and *MB-COMT*. Gibbs free energy (Δ*G*) for the stem-loop structure associated with the HPS haplotype is ~17 kcal/mol less than that associated with the LPS haplotype for both *S-COMT* and *MB-COMT* (Fig. 1B and fig. S1A). Additional evidence supporting predicted RNA folding structures was obtained by generating consensus RNA secondary structures based on comparative analysis of *COMT* sequences from eight mammalian species (fig. S2). The consensus RNA folding structures were LPS-like and did not contain highly stable local stem-loop structures analogous to the human HPS-like form. Thus, substantial deviation from consensus structure, as observed for the HPS haplotype, should have notable functional consequences. Additional studies were conducted to test this molecular modeling.

We constructed full-length S- and MB-COMT cDNA clones in mammalian expression vectors that differed only in three nucleotides corresponding to the LPS, APS, and HPS haplotypes (*17*, *21*). Rat adrenal (PC-12) cells were transiently transfected with each of these six constructs. COMT enzymatic activity, protein expression, and mRNA abundance were measured. Relative to the LPS haplotype, the HPS haplotype showed a 25- and 18-fold reduction in enzymatic activity for S- and MB-COMT constructs, respectively (Fig. 1C and fig. S1B). The HPS haplotype also exhibited marked reductions in S- and MB-COMT protein expression (Fig. 1D and fig. S1C). The APS haplotype displayed a moderate 2.5- and 3-fold reduction in enzymatic activity for S- and MB-COMT constructs, respectively, while pro-

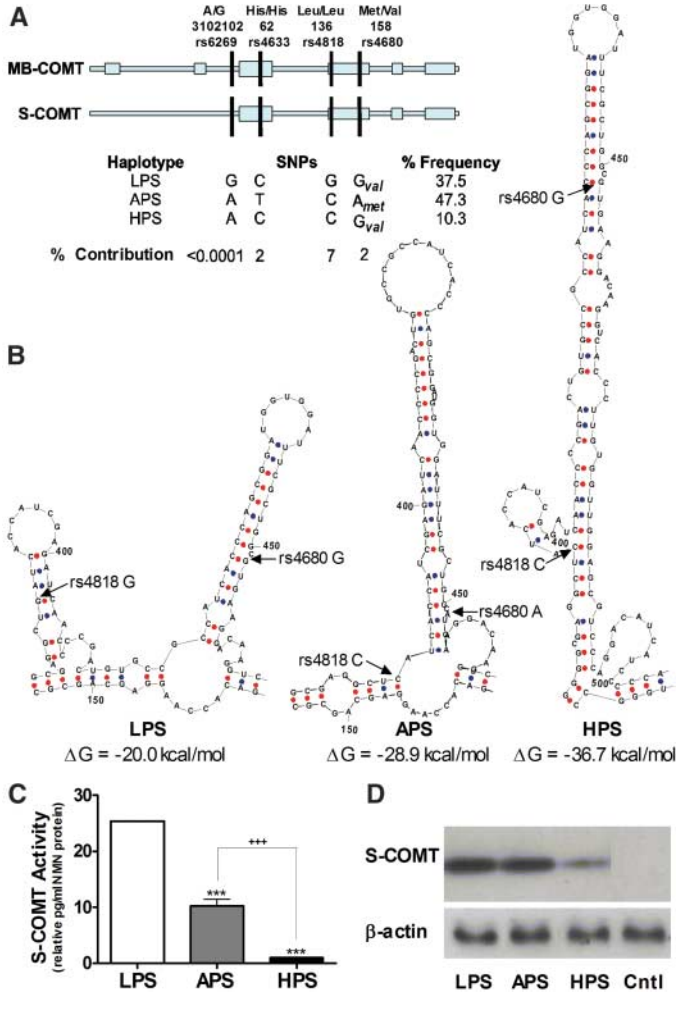

Fig. 1. Common haplotypes of the human *COMT* gene differ with respect to mRNA secondary structure and enzymatic activity. (**A**) A schematic diagram illustrates *COMT* genomic organization and SNP composition for the three haplotypes. Percent frequency of each haplotype in a cohort of healthy Caucasian females, and percent independent SNP contribution to pain sensitivity, are indicated. (**B**) The local stem-loop structures associated with each of the three haplotypes are shown. Relative to the LPS and APS haplotypes, the HPS local stem-loop structure had a higher folding potential. (**C** and **D**) The LPS haplotype exhibited the highest, while the HPS haplotype exhibited the lowest enzymatic activity and protein levels in cells expressing COMT. $^{***}P < 0.001$, ≠ LPS. $^{+++}P < 0.001$, ≠ APS.

tein expression levels did not differ. The moderate reduction in enzymatic activity produced by the APS haplotype is most likely due to the previously reported decrease in protein thermostability coded by the *met*^{158} allele (*3*). These data illustrate that the reduced enzymatic activity corresponding to the HPS haplotype is paralleled by reduced protein levels, an effect that could be mediated by local mRNA secondary structure at the level of protein synthesis and/or mRNA degradation. Because total RNA abundance and RNA degradation rates did not parallel COMT protein levels (fig. S2), differences in protein translation efficiency likely results from differences in the local secondary structure of corresponding mRNAs.

To directly assess this hypothesis, we performed site-directed mutagenesis (*17*). The stable stem-loop structure of *S-* and *MB-COMT* mRNA corresponding to the HPS haplotype is supported by base pairs between several critical nucleotides, including 403C and 479G in *S-COMT* and 625C and 701G in *MB-COMT* (Fig. 2A and fig. S3A). Mutation of 403C to G in *S-COMT* or 625C to G in *MB-COMT* destroys the stable stem-loop structure and converts it into a LPS haplotype–like structure (HPS Lsm). Double mutation of mRNA in position 403C to G and 479G to C in *S-COMT* or 625C to G and 701G to C in *MB-COMT* reconstructs the original long stem-loop structure (HPS dm). The single- and double-nucleotide HPS mutants (HPS Lsm and HPS dm, respectively) were transiently transfected to PC-12 cells. As predicted by the mRNA secondary-structure folding analyses, the HPS Lsm exhibited increased COMT enzymatic activity and protein levels equivalent to those of the LPS haplotype, whereas the HPS dm exhibited reduced enzymatic activity and protein levels equivalent to those of the original HPS haplotype (Fig. 2, B and C; fig. S3, B and C). These data rule out the involvement of RNA sequence recognition motifs or codon usage in the regulation of translation. In contrast to the HPS haplotype, protein levels did not parallel COMT enzymatic activity for the APS haplotype and site-directed mutagenesis confirmed that the *met*^{158} allele, not a more stable mRNA secondary structure, drives the reduced enzymatic activity observed for the APS haplotype (fig. S4). This difference is moderate relative to the mRNA structure–dependent difference coded by LPS and HPS haplotypes. These results were verified by an alternate approach of modifying mRNA secondary structure (fig. S5).

Our data have very broad evolutionary and medical implications for the analysis of variants common in the human population. The fact that alterations in mRNA secondary structure resulting from synonymous changes have such a pronounced effect on the level of protein expression emphasizes the critical role of synonymous nucleotide positions in maintaining mRNA secondary structure and suggests that the mRNA secondary structure, rather than independent nucleotides in the synonymous positions, should undergo substantial selective pressure (*22*). Furthermore, our data stress the importance of synonymous SNPs as potential functional variants in the area of human medical genetics. Although nonsynonymous SNPs are believed to have the strongest impact on variation in gene function, our data clearly demonstrate that haplotypic variants of common synonymous SNPs can have stronger effects on gene function than nonsynonymous variations and play an important role in disease onset and progression.

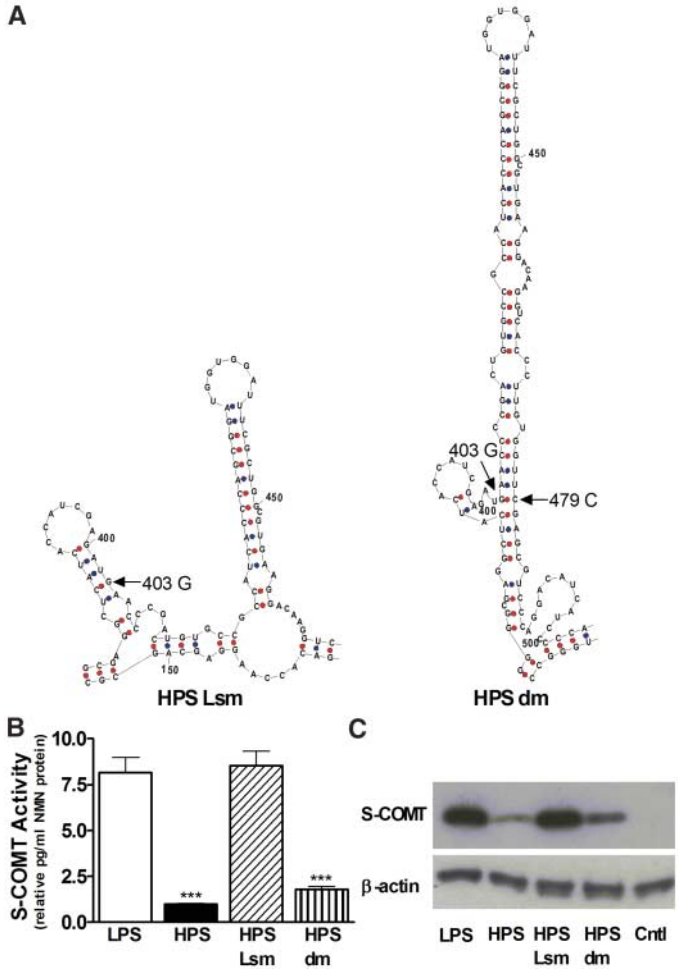

Fig. 2. Site-directed mutagenesis that destroys the stable stem-loop structure corresponding to the HPS haplotype restores COMT enzymatic activity and protein expression. (**A**) The mRNA structure corresponding to the HPS haplotype was converted to an LPS haplotype-like structure (HPS Lsm) by single mutation of 403C to G. The original HPS haplotype structure (HPS dm) was restored by double mutation of interacting nucleotides 403C to G and 479G to C. (**B** and **C**) The HPS Lsm exhibited COMT enzymatic activity and protein levels equivalent to those of the LPS haplotype, whereas the HPS dm exhibited reduced enzymatic activity. ***$P < 0.001$, ≠ LPS.

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Lineages of Acidophilic Archaea Revealed by Community Genomic Analysis

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Novel, low-abundance microbial species can be easily overlooked in standard polymerase chain reaction (PCR)–based surveys. We used community genomic data obtained without PCR or cultivation to reconstruct DNA fragments bearing unusual 16*S* ribosomal RNA (rRNA) and protein-coding genes from organisms belonging to novel archaeal lineages. The organisms are minor components of all biofilms growing in pH 0.5 to 1.5 solutions within the Richmond Mine, California. Probes specific for 16*S* rRNA showed that the fraction less than 0.45 micrometers in diameter is dominated by these organisms. Transmission electron microscope images revealed that the cells are pleomorphic with unusual folded membrane protrusions and have apparent volumes of <0.006 cubic micrometer.

Our understanding of the variety of microorganisms that populate natural environments was advanced by the development of polymerase chain reaction (PCR)–based, cultivation-independent methods that target one or a small number of genes (*1*–*3*). Genomic analyses of DNA sequence fragments derived from multispecies consortia (*4*–*6*) and whole environments (*7*–*9*) have provided new information about diversity and metabolic potential. However, PCR-based methods have limited ability to detect organisms whose genes are significantly divergent relative to gene sequences in databases, and most cultivation-independent genomic sequencing approaches are relatively insensitive to organisms that occur at low abundance. Consequently, it is likely that low-abundance microorganisms distantly related to known species will be undetected members of natural consortia, even in low complexity systems such as acid mine drainage (AMD) (*10*).

An important way in which microorganisms affect geochemical cycles is by accelerating the dissolution of minerals. For example, microorganisms can derive metabolic energy by oxidizing iron released by the dissolution of pyrite (FeS_2). The ferric iron by-product promotes further pyrite dissolution, leading to AMD generation. AMD solutions forming underground in the Richmond Mine at Iron Mountain, California, are warm (30° to 59°C), acidic (pH ~0.5 to 1.5), metal-rich [submolar Fe^{2+} and micromolar As and Cu (*11*)] and host active microbial communities. Extensive cultivation-independent sequence analysis of functional and rRNA genes (*11*, *12*) revealed that biofilms contain a significant number of Archaea, but the diversity reported to date has been limited to the order Thermoplasmatales (*10*). Current models for AMD generation thus include only these species.

The genomes of the five dominant members of one biofilm community from the "5-way" region of the Richmond Mine (fig. S1) were largely reconstructed through the assembly of 76 Mb of shotgun genomic sequence (*4*). Previously unreported is a genome fragment that encodes part of the 16*S* rRNA gene of a novel archaeal lineage: Archaeal Richmond Mine Acidophilic Nanoorganism (ARMAN-1). Using an expanded data set that now comprises more than 100 Mb of genomic sequence, we reconstructed a contiguous 4.2-kb fragment adjacent to this gene. A second 13.2-kb genome fragment encoding a 16*S* rRNA gene from an organism that is related to ARMAN-1 (ARMAN-2) was reconstructed from 117 Mb of community genomic sequence derived from a biofilm from the A drift (fig. S1). Within the data sets from each site, results to date indicate that each ARMAN population is near-clonal.

Comparison of the ARMAN-1 and -2 DNA fragments revealed some gene rearrangements, insertions, and deletions (Fig. 1). Genes present in both organisms encode putative inorganic pyrophosphatases, a transcription regulator, and a gene shown to be an arsenate reductase (*13*). Comparative analysis of these genes with sequences in the public databases consistently

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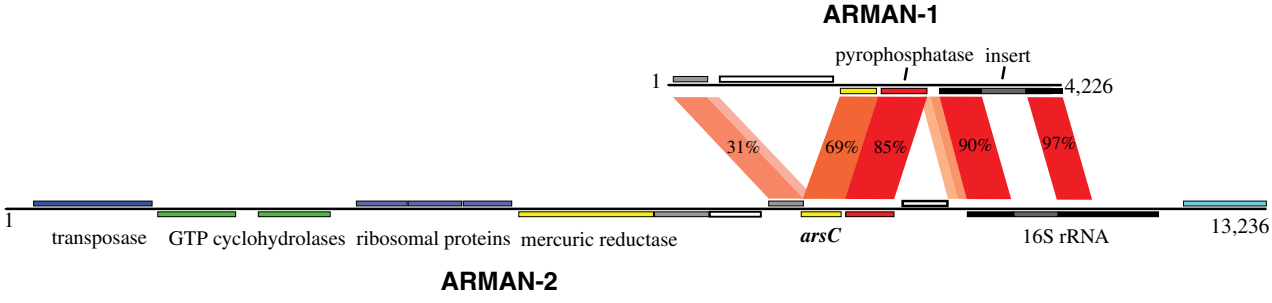

Fig. 1. Comparison of syntenous genomic regions of ARMAN-1 [from the "5-way" (CG) community (*4*)] and ARMAN-2 (from the UBA community). Orthologs and their protein identity are indicated by the red bands. The percentage similarity for the 16*S* rRNA gene sequences is also shown. Numbers at ends indicate length (number of nucleotides); predicted open reading frames for hypothetical proteins are indicated by boxes.

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