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Genotypic Diversity within a Natural Coastal Bacterioplankton Population

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ABSTRACT:

The genomic diversity and relative importance of unique genotypes within natural bacterial populations has remained largely unknown. Here, we analyze the diversity and annual dynamics of a group of coastal bacterioplankton (>99% 16S rRNA identity to *Vibrio splendidus*). We show that this group consists of at least a thousand distinct genotypes, each occurring at extremely low environmental concentrations (on average <1 cell/ml). Overall, the genomes show extensive allelic diversity and size variation. Individual genotypes rarely recurred in samples and allelic distribution did not show spatial or temporal substructure. Ecological considerations suggest that much genotypic and possibly phenotypic variation within natural populations should be considered neutral.

Molecular evidence increasingly demonstrates the remarkable genetic diversity of the microbial world (*1, 2*) yet ecological interpretation of this diversity remains elusive. This is largely because microbiologists rely on studies of clonal isolates or environmental gene libraries to infer biogeochemical and pathogenic functions of natural bacterial populations. What is missing, however, is quantitative information regarding the environmental prevalence of individual genotypes that would allow inference of their ecological significance or competitive success. On the one hand, it may be expected that ecologically distinct populations display relatively high clonality because bacterial genomes have a high potential for adaptive mutations, which may lead to purging of genotypic diversity from within the population by selective sweeps (*3, 4*). On the other hand, this view is increasingly difficult to reconcile with recent observation of high levels of differentiation among closely related genomes [e.g. (*5, 6*)] and the recovery of vast numbers of similar but non-identical homologous genes from environmental samples (microdiversity) (*7-9*). It may therefore be questioned whether competition among individual strains is strong enough to result in frequent selective sweeps or instead whether natural populations may accumulate large neutral allelic and perhaps even genomic variation (*8*). However, the diversity and prevalence of individual variants within environmental bacterial populations has not been extensively explored and so questions regarding the ecological significance of genotypic variation remain unanswered.

To analyze genotypic diversity and overall population size quantitatively, we combined culture-dependent and independent methods to assess the number, extent of variation, and

relative frequency of genotypes within a well-defined natural bacterial population. We chose a coastal assemblage of *Vibrio splendidus*, previously identified as a phylogenetically discrete cluster denoted by nearly identical (<1% divergent) 16S rRNA sequences in an analysis of bacterioplankton community structure (*8*). We have proposed that such ribotype clusters represent ecologically differentiated units, i.e., ecotypes or populations (*8*). Thus, we defined the *V. splendidus* cluster as a population of naturally co-occurring genomes that can be tracked quantitatively in the environment and identified in strain collections by their distinct rRNA genes.

Quantification of the *V. splendidus* population over an annual cycle by QPCR (*10*) revealed that it is consistently present as a member of the coastal bacterioplankton community and displays seasonal variation in abundance (Fig. 1A). Concomitant with quantification, we isolated strains from five temporal samples on *Vibrio*-selective media and identified strains by 16S rRNA sequence analysis (Fig. 1B) (*11*). Overall, 20 distinct *Vibrio* (and closely related *Photobacterium*) taxa grew on the media (Fig. 1B and C) but the majority of isolates (232 of 333) were identified as members of the *V. splendidus* population (red sectors in Fig. 1B). This dominance in all collections, except the cold-water sample (March 03) (Fig. 1B), roughly parallels the culture-independent quantification by QPCR (Fig. 1A).

Determination of sequence diversity of a universally distributed protein-coding gene (Hsp60) among all 333 *Vibrio* isolates showed high heterogeneity but confirmed the monophyly of the *V. splendidus* population detected by the rRNA sequence analysis (data

not shown). We observed 141 unique Hsp60 alleles among the 232 *V. splendidus* isolates (Fig. 2), and extrapolation using the Chao-1 richness estimator (*12*) suggests a minimum of 436 alleles in the total sampled volume (31.5 ml). Despite these high numbers, the Hsp60 sequences collapse into a single group at 95% nucleotide consensus (Fig. 2 and S1) with variation primarily limited to neutral third codon positions (average K_A/K_S = 0.04). No single allele showed clear dominance among the isolates suggesting a relatively even abundance of the strains (Fig. S1) and analysis of molecular variance (AMOVA) did not reveal evidence for population structure associated with temporal (one month to one year) and spatial (100 μl to 2 ml) scales.

Still greater heterogeneity was revealed within the *V. splendidus* population when the number of unique genotypes among the cultured strains was assayed by pulse field gel electrophoresis (PFGE). PFGE analysis detected 180 unique genome patterns among the 206 strains tested; demonstrating that the majority of isolates possess distinct genomes (Fig. S1). The Chao-1 estimator (*12*) yielded a total of 1,287 unique genotypes in the samples, considerably exceeding the estimated allelic diversity of the Hsp60 gene. Moreover, the PFGE analysis provides evidence that, in addition to accumulation of point mutations as observed for the Hsp60 alleles, a large proportion of genotypes are differentiated by insertions and deletions of large genome fragments. This was suggested by variation among strains in the sum of genome fragment sizes in the PFGE gels and was explored in detail for a set of 12 strains. Pairs of isolates with identical Hsp60 alleles were chosen so that sequence identities between pairs reflected overall divergence in these genes (Fig. 3). Among these 12 isolates, genome sizes ranged from 4.5 to 5.6 Mb

with only weak correlation of genome size difference to Hsp60 sequence divergence (R = 0.37) and only minor contribution to size estimates by plasmids (data not shown). Even when comparing strains with identical Hsp60 alleles, most (four of the six pairwise comparisons) showed significant genome size differences (Student's t-test, $p<0.01$; ~170 to 800 kb variation) indicating that specific alleles may be poor markers for distinct genomes.

The high degree of heterogeneity observed among the *V. splendidus* genomes suggests that the average concentration of individual genotypes is astoundingly small in the sampled environment. To illustrate this, we divided the QPCR-based estimates of population size of *V. splendidus* in samples taken in Aug 03, Sept 03, and Oct 03 (1,890, 600, and 640 cells/ml, respectively) (Fig. 1A) by the Chao-1 estimates for the number of Hsp60 alleles (125, 94 and 279) and genotypes (465, 553 and 901) in those same samples. The result suggests that unique Hsp60 alleles occurred in the monthly samples at average concentrations of 2 to 15 cells per ml (or at a frequency of 0.3 to 1%) while unique genotypes were present at ~10-fold lower frequency (average concentration for all samples estimated at <1 cell per ml). If the possibility of isolation bias is taken into account the estimated concentrations (population size/richness) would be even lower; because isolation bias would lead to an underestimation of richness but would not affect population size estimates by QPCR.

What could explain such high diversity of *V. splendidus* genotypes in this environment? The observed pattern suggests that purging of genotypes from within the population

(operationally defined as a ribotype cluster) is rare compared to processes introducing variation, and that variation persists because it is either favored by selection (e.g. by balancing selection or niche differentiation) or is neutral. Indeed, some proportion of the observed genotypic diversity may reflect the differentiation of (sub)populations that are specialized to particular environmental conditions in the complex life-style of vibrios (including free-living and animal or particle associated states). However, ecological considerations suggest that much of the observed genotypic diversity has little adaptive significance in the context of the water column. Given their low estimated concentration (<1 cell/ml), individual genotypes would occupy much less than a trillionth of the volume of a ml of seawater. Because resources are thought to arise in small patches that are unpredictable relative to the location of any given cell (*13*), access by unique genotypes to conditions allowing rapid growth may be largely stochastic, relegating strong competitive interactions between genotypes to ephemeral microzones. In addition, top-down interactions, like predation (*14*), may quickly erase any localized dominance of genotypes. Thus, although individual genotypes may achieve rapid growth in microzones or microcolonies, averaged over the water column their differences do not result in lasting growth advantage (i.e., they are effectively neutral) and so the observed vast genotypic diversity can coexist.

Previous studies have shown that significant variation in gene content and genome size may occur among closely related genomes (albeit drawn from separate environments) (*6, 15-17*). Such variation can arise via gene duplication, insertion, and deletion or by horizontal gene transfer (HGT) mediated by phages, plasmid-borne transposons and

integrons (*18*). In fact, HGT is now regarded as a major source of innovation in bacterial evolution (*19-21*) and several cases of environmental differentiation have been linked to specific gene addition or loss (*22-24*). However, it has also been suggested that most acquired sequences do not confer a selective advantage on their host and can be neutral targets for deletion and mutational events leading to a dynamic genome (*25, 26*). Indeed, model results indicate that such neutral genome segments are likely to be transient elements represented in only a small fraction of a population (*27*). Our results expand such previous considerations to indicate that large genome modifications, possibly including HGT, are observed with high frequency in genomes that contain identical rRNA and Hsp60 sequences and coexist within the same natural population.

It will be important to ask whether such extensive genomic variation is a general feature of natural bacterial populations. We have recently observed that two microbial communities are composed of hundreds of microdiverse ribotype clusters (*8, 9*) and have proposed that these denote bacterial populations, which arise by rare selective sweeps followed by effectively neutral diversification (*8*). Consistent with this hypothesis, we show that one such cluster occurs predictably in the bacterioplankton community and contains extensive diversity, much of which may be neutral in the ecological context of the water column. If similar patterns of diversity are common to bacterial communities caution should be exercised in interpreting the extent to which gene complements or even metabolic traits of individual isolates may reflect the overall properties of populations (*28-30*). Indeed our results suggest that not only the gene content, but also quantitative

abundance and dynamics of individual traits should be considered when evaluating the ecological significance of differences among coexisting genotypes.

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Foundation. Sequences have been submitted to Genbank under accession numbers AY836800-AY837464.

Supporting Online Material

www.sciencemag.org

Materials and Methods

Fig. S1

Figure legends

Fig. 1. Diversity and abundance of coastal vibrioplankton (Plum Island Sound, MA) in monthly samples taken over an entire year. **(A)** Quantification of *V. splendidus* (red) and total vibrio (black) populations by culture independent QPCR. **(B)** Relative proportion of *Vibrio* and *Photobacterium* isolates by phylogenetic association. Color-coding as in (C). **(C)** Phylogenetic relationships among representative *Vibrio* and *Photobacterium* isolates inferred from distance analysis of partial 16S rRNA sequences (bootstrap proportions >50% are indicated above nodes).

Fig. 2. Number of unique Hsp60 clusters among *V. splendidus* isolates observed as cluster cutoff values are decreased from 100 to 95%.

Fig. 3. Genome size estimates and phylogenetic relationships of Hsp60 sequences for 12 *V. splendidus* isolates chosen as pairs with identical Hsp60 alleles, encompassing all levels of Hsp60 variation observed in the strain collection. **(A)** Genome sizes determined by PFGE as averages of six independent estimates, each obtained from single enzyme digests run to resolve large, medium and small-sized bands, respectively, and repeated three times for each of two enzymes (NotI/SfiI or NotI/AscI) per isolate. **(B)** Phylogenetic relationships of Hsp60 alleles inferred from maximum likelihood analysis with assumption of molecular clock from partial gene sequences. Isolate identifiers correspond to month (12 = 8/12/03; 13 = 9/10/03; 14 = 10/11/03) of isolation and strain name.

Figure 1

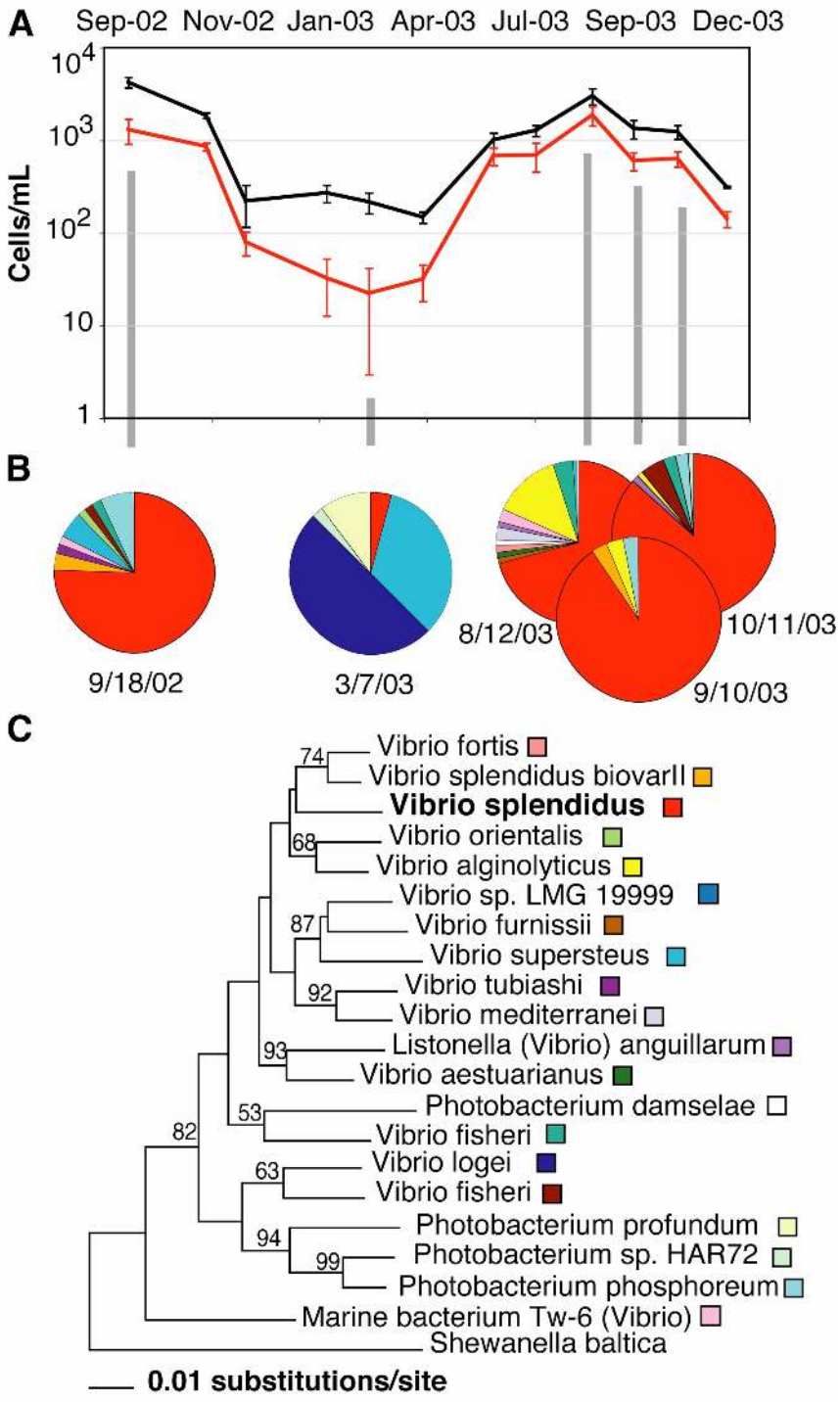

A
Sep-02 Nov-02 Jan-03 Apr-03 Jul-03 Sep-03 Dec-03
Cells/mL
10^4
10^3
10^2
10
1
B
9/18/02
3/7/03
8/12/03
10/11/03
9/10/03
C
Vibrio fortis
Vibrio splendidus biovarII
Vibrio splendidus
Vibrio orientalis
Vibrio alginolyticus
Vibrio sp. LMG 19999
Vibrio furnissii
Vibrio supersteus
Vibrio tubiashi
Vibrio mediterranei
Listonella (Vibrio) anguillarum
Vibrio aestuarianus
Photobacterium damselae
Vibrio fisheri
Vibrio logei
Vibrio fisheri
Photobacterium profundum
Photobacterium sp. HAR72
Photobacterium phosphoreum
Marine bacterium Tw-6 (Vibrio)
Shewanella baltica
74
68
87
92
93
53
82
63
94
99
0.01 substitutions/site

Figure 2

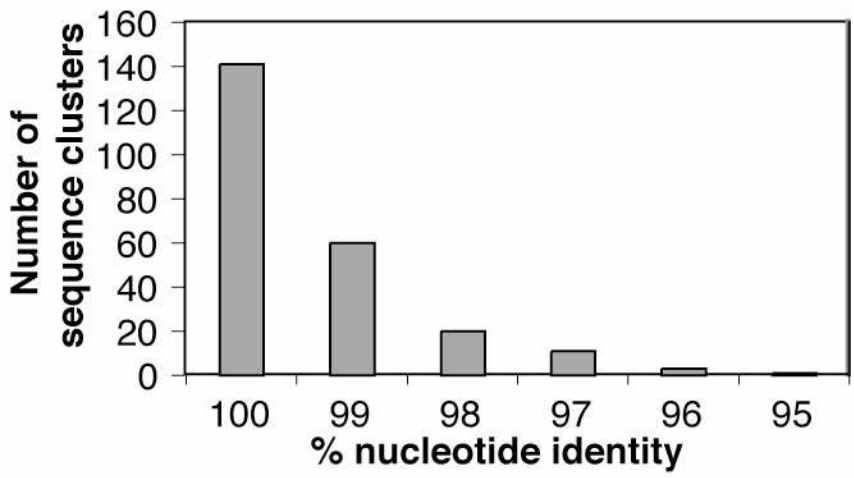
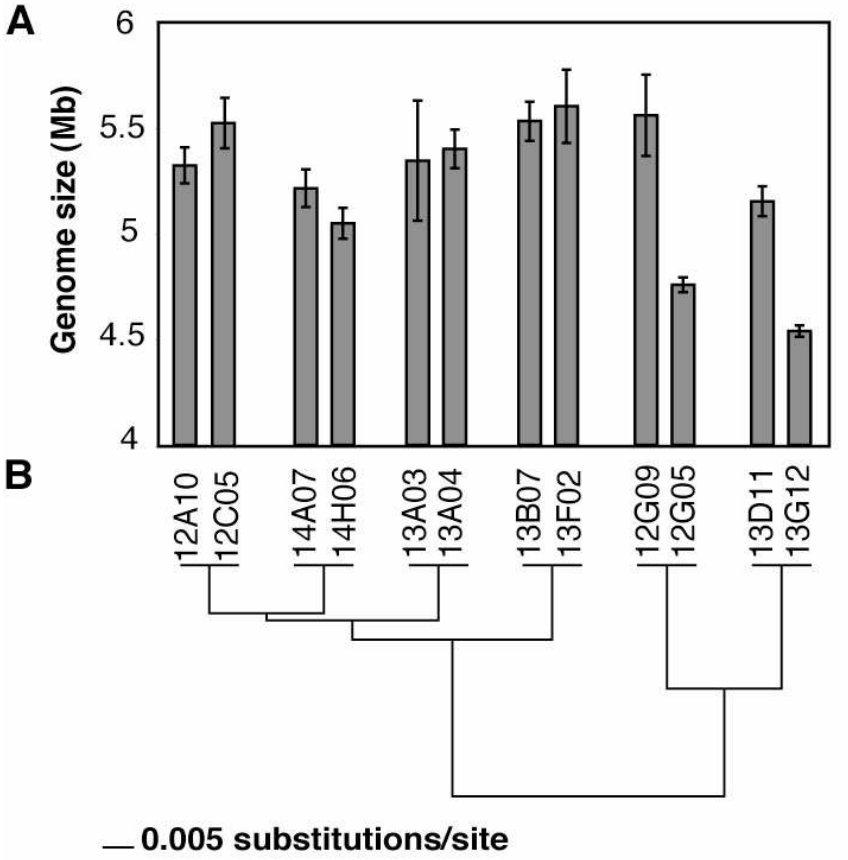

Number of sequence clusters
160
140
120
100
80
60
40
20
0
100
99
98
97
96
95
% nucleotide identity

Figure 3


A
6
5.5
5
4.5
4
Genome size (Mb)
B
12A10
12C05
14A07
14H06
13A03
13A04
13B07
13F02
12G09
12G05
13D11
13G12
0.005 substitutions/site

SUPPLEMENTAL ONLINE MATERIAL

Materials and Methods

Study Site, sampling and strain isolation.

Samples for strain isolation and nucleic acid extraction were collected monthly at high tide from the marine end of the Plum Island Sound estuary (northeastern Massachusetts) (*1*). Strains were isolated by subsampling each of four 1-liter water samples by direct plating onto TCBS (Difco) agar plates containing 2% NaCl. From the 9/18/02 samples, aliquots of 0.1 ml were plated directly; for all other samples, bacterioplankton was concentrated on filters (0.2 μm) (Supor); these were placed onto plates and incubated at room temperature. For samples taken on 8/12/03, 9/10/03 and 10/11/03 aliquots of 100 μl to 2 ml were concentrated, while for 3/7/03, 10 to 20 ml were used. The total volumes sampled were 12.1, 8, 8, and 140 ml for 8/12/03, 9/10/03, 10/11/03 and 3/7/03, respectively. Single colonies were picked and purified by serial subculture onto TCBS and 2216 agar (Difco) and were stored in glycerol at –80°C. For culture-independent estimation of total vibrioplankton and *V. splendidus* abundance (*2*), bacterioplankton was concentrated onto duplicate 0.2 μm filters (Sterivex or Poretics) in aliquots of 300 to 700 ml and stored at –20°C. Water temperatures for the dates of strain isolation were (16, 16, 16, 13.5 and 1.5˚ C respectively) and salinity was between 30-33 ppt.

Estimation of Population Abundance.

Nucleic acids were extracted from filter-concentrated bacterioplankton with a bead-beating method (*2*). The abundance of *V. splendidus* was determined by QPCR with *Vibrio*-specific 16S rRNA primers using a competitive internal standard, followed by separation and quantification of the resulting amplicons by constant denaturing capillary electrophoresis (CDCE) (*2*). *V. splendidus* populations were identified as amplicons co-migrating in CDCE spectra with amplicons from *V. splendidus* isolates obtained from the study site and from cloned 16S rRNA genes obtained from a previous investigation (*2*). Total vibrioplankton abundance was obtained by summing all *Vibrio*-specific CDCE-peaks observed (*2*). Cell numbers were inferred based on an average of 9 rRNA operons per genome for *V. splendidus* determined by Southern blot analysis of several strains; for total vibrioplankton the average operon number for the genus *Vibrio* was used (*2*).

DNA extraction from strains and sequence analysis.

DNA from bacterial isolates was purified using the PureGene kit (Gentra Systems). The 16S rRNA and Hsp60 genes were PCR amplified using conditions described previously (*2*) with primers 27F and 1492R (*3*), and H279 and H280 (*4*), respectively. Sequences were determined using primers 27F and 789R (16S rRNA) (*3*), and H279 and H280 (Hsp60). All unique alleles were confirmed by reamplification and sequencing. Neither the 16S rRNA nor the Hsp60 gene fragments of the *V. splendidus* strains contained any length heterogeneity resulting in unambiguous alignments of 723 and 541 nucleotides, which were used for all further analysis. The 16S rRNA sequences contained multiple, clustered ambiguities, which likely stem from sequence heterogeneity among the 8-10 rRNA operons we have detected by Southern hybridization of

representative isolates (data not shown). However, overall sequence divergence remained <1% even when ambiguities were counted as differences. This is, consistent with previous analysis of operon heterogeneity in published genomes (*5*).

Sequences were compared to establish relationships and test population substructure in the samples. Percent sequence similarity groups of 16S rRNA and Hsp60 alleles were calculated using Clusterer (*1*). All phylogenetic analyses were performed using programs contained in PAUP* (Phylogenetic Analysis Using Parsimony) [provide full citation] (*6*). Neighbor Joining trees were used as a preliminary tool to identify microdiverse ribotype clusters (with roughly ≥99% internal 16S rRNA sequence identity) from among all 333 *Vibrio* and (closely-related) *Photobacterium* isolates. The resulting clusters were named according to the closest sequence identified by Seqmatch implemented in the RDPII (Ribosomal Database Project) website (*7*). A representative subtree, including one sequence from each cluster, was constructed using Distance methods with Jukes-Cantor correction and 100 Bootstrap replicates. Relationships among Hsp60 alleles from strains used for detailed genome size determination were inferred by maximum-likelihood (PAUP, default parameters except with molecular clock enforced), and 100 Bootstrap replicates were performed. The ratio of nonsynonymous to synonymous mutations (K_A/K_S) among Hsp60 sequences was determined using DNASP v. 4.0 (*8*). For each sampling date, and for all sampling dates combined, three hierarchical components of Hsp60 allelic diversity (within date(s), within date(s) among sub-samples and within sub-samples) were tested using Analysis of Molecular Variance (AMOVA) [implemented in Arlequin 2.0 (*9*)]. For estimation of allelic and genotypic richness in the samples, the Chao-1 non-parametric richness estimator was used (*10, 11*) because it does not assume a specific abundance distribution model.

Genotypic diversity, genome size determination and identification of plasmids.

Pulsed field gel electrophoresis (PFGE) was used as a robust method for determining whole genome differentiation (genotyping) and genome sizes. All digests were run using the Chef II and Chef mapper (Biorad) and analyzed using the Gel Compar II software package (Biomathematica). For genotyping, Not I digests of whole genomic DNA were separated (6V/cm with a 5.3 to 34.9 second switch time for 19 hours at 14˚C) and 50-500kb fragments were normalized to the lambda ladder (BioRad) and compared to each other. Patterns were replicated for ~25% of the strains. RAPD-PCR with primers OPD11 and OPD20 (*12*) was used as an independent genotyping method to confirm PFGE results and also for routine checks of strain identity. Genome sizes were determined for 12 strains. These were selected to form six pairs such that within pairs Hsp60 alleles were identical while between pair differences reflected the total range of divergence observed among strains. Genome sizes were determined as averages of six independent estimates, each obtained from single enzyme digests run using conditions optimized to resolve large, medium and small-sized bands and repeated three times for each of two enzymes (NotI/SfiI or NotI/AscI) per genome. Fragments >500kb, between 500 and 50, and <50kb were sized using conditions recommended to resolve the *Saccharomyces cerevisae* ladder (BioRad), lambda ladder (BioRad), and the low molecular weight PFGE ladder (New England Biolabs), respectively. Co-migrating bands not resolved under the three PFGE conditions were identified by band intensity. For

detection of plasmid DNA, alkaline lysis preps were performed to exclude genomic DNA and these were resolved on agarose gels (*13*).

Supplemental References

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Fig. S1 Comparison of allelic and genotypic variation among *V. splendidus* strains with each row denoting a unique strain. **(A)** Hsp60 alleles grouped by increasing sequence identity cut-off values (95 to 100%, left to right). Colored boxes delineate sequence identity groups (strains with alleles not differentiated at the specified cut-off value). White boxes identify unique alleles at the specified cut-off value. **(B)** Genotypes identified by PFGE profiles arranged to match corresponding Hsp60 alleles. Strains from which no PFGE pattern could be obtained are indicated by X. Note that strains with identical Hsp60 alleles frequently have different PFGE patterns; however, strains with identical PFGE patterns almost always have identical Hsp60 allele.