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Uncovering major genomic features of essential genes in Bacteria and a methanogenic Archaea

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Abstract

Identification of essential genes is critical to understand the physiology of a species, propose novel drug targets and uncover minimal gene sets required for life. Although essential gene sets of several organisms have been determined using large-scale mutagenesis techniques, systematic studies addressing their conservation, genomic context and functions remain scant. Here we integrate 17 essential gene sets from genome-wide *in vitro* screenings and three gene collections required for growth *in vivo*, encompassing 15 Bacteria and one Archaea. We refine and generalize important theories proposed using *Escherichia coli*. Essential genes are typically monogenic and more conserved than nonessential genes. Genes required *in vivo* are less conserved than those essential *in vitro*, suggesting that more divergent strategies are deployed when the organism is stressed by the host immune system and unstable nutrient availability. We identified essential analogous pathways that would probably be missed by orthology-based essentiality prediction strategies. For example, *Streptococcus sanguinis* carries horizontally-transferred isoprenoid biosynthesis genes that are widespread in Archaea. Genes specifically essential in *Mycobacterium tuberculosis* and *Burkholderia pseudomallei* are reported as potential drug targets. Moreover, essential genes are not only preferentially located in operons, but also occupy the first position therein, supporting the influence of their regulatory regions in driving transcription of whole operons. Finally, these important genomic features are shared between Bacteria and at least one Archaea, suggesting that high order properties of gene essentiality and genome architecture were probably present in the last universal common ancestor or evolved independently in the prokaryotic domains.

Graphical Abstract

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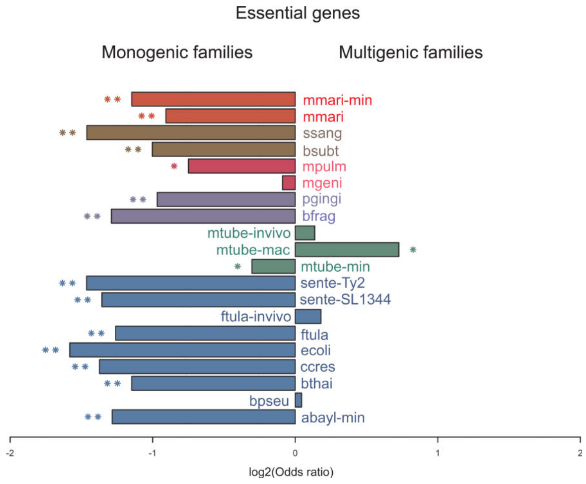
AUTHOR CONTRIBUTIONS
Conceived the study: ALG, NMV, TMV; planned the analyses: ALG, NMV, TMV; performed the analyses: ALG, NMV, TMV; Wrote the paper: ALG, NMV, TMV.



Keywords

essential genes; operons; genome organization; prokaryotes; transposon mutagenesis; genome evolution

INTRODUCTION

Bacteria and Archaea are widely diversified prokaryotic domains [1], adapted to a wide range of niches [2]. Prokaryotes evolved over billions of years and divergence of the major groups of Bacteria and of Archaea occurred between 2.5-3.2 and 3.1-4.1 billion years, respectively [3]. Prokaryotic genomes have been carved by selection pressures, population size bottlenecks, mutation and recombination rates and mobile genetic elements [4], resulting in highly variable genome sizes and contents in different phylogenetic groups [5, 6]. Genome sequencing efforts over the past two decades fueled the search for a universal set of genes that would represent the minimal genome. However, it has been demonstrated that as phylogenetic distance increases, the number of universal genes is reduced to a level that is unlikely to support cellular life [7, 8]. Single-gene deletion techniques and modern approaches such as large-scale transposon mutagenesis followed by high-throughput sequencing allowed simultaneous screening of as many as 1 million mutants with high-resolution [9-11]. Determination of bacterial essential genes using these approaches improved gene annotations [9], mapping of metabolic pathways [10, 12], identification of genotype to phenotype associations [12] and helped to define genome-wide essential gene sets *in vitro* [13] and during infection and colonization [14, 15]. As gene essentiality is condition-dependent, *in vivo* screenings have gained attention [15-17] because of their potential to uncover genes involved in pathogenesis, which are of particular interest in the case of resistant bacteria [9, 13]. Moreover, identification of essential genes is critical for the development of engineered cells for compound production [18].

Integrative analyses of computationally and experimentally-determined essential gene sets uncovered important features that constitute the basis of essentiality prediction in bacteria, such as their lower substitution rates when compared to nonessential genes [19] and the correlation of essentiality with high gene expression [20] and conservation [21]. Functional analysis using Clusters of Orthologous Groups (COGs) [22] revealed that *Information storage and processing* genes are overrepresented among essential genes in most species, whereas species specificity was found in more peripheral metabolic pathways [23]. Moreover, only 34% and 61% of the *Bacillus subtilis* and *Escherichia coli* essential genes are universally conserved in their phyla (Firmicutes and Gamma-proteobacteria, respectively) [21], favoring essentiality prediction based on persistence, according to which persistent genes are those shared by most genomes [21]. Persistence analysis allowed the identification of truly essential genes that are frequently missing in essential gene sets (e.g. DNA repair genes) [21, 24]. Further, essential and persistent nonessential genes share common characteristics such as: high sequence conservation and expression rates (predicted by the codon adaptation index); preferential localization at the chromosomal leading strand, minimizing the risk of head-on collisions between DNA and RNA polymerases [20], and tendency to be in operons [21]. Approximately 60% of the bacterial genes are co-transcribed in polycistronic RNAs derived from operons [25]. The most accepted theory of operon formation is the co-regulation hypothesis [26, 27], which postulates that operons are formed by rearrangements that place two or more genes together, with subsequent maintenance of such structure by selection for concerted transcriptional regulation and translation of functionally related proteins. Further, according to the co-regulation hypothesis, essential genes would be preferentially located in operons, as observed in *E. coli* [26, 27].

Although important discoveries have been reported by means of comparative genomics and experimental data on gene essentiality in *E. coli*, the progress brought upon by next-generation sequencing and mutagenesis methods allowed the evaluation of distantly related species with high resolution. Here we integrate data from 20 genome-wide screenings in 16 organisms, encompassing 17 saturated *in vitro* and 3 *in vivo* datasets. Unlike previous studies, we took advantage of a recently published archaeal essential gene set [28], extending our analyses to the two prokaryotic domains of life. Important trends discovered in *E. coli* are also present in all major bacterial groups, such as the extensive conservation of essential genes and their propensity to be in operons. Moreover, essential gene sets *in vitro* are very different from those *in vivo*, probably because of the recruitment of a less conserved gene set to survive under stress conditions and limited nutrient sources. We have also demonstrated that essential genes do not only tend to be in operons, but also occupy the first position therein. Finally, many genomic features of the bacterial essential genes are also present in the only archaeal species for which a large-scale essentiality screening is available, suggesting that a high level genomic organization could have been either present in the last universal common ancestor (LUCA) or evolved independently in the two prokaryotic domains of life.

RESULTS AND DISCUSSION

The number of essential genes is not correlated to genome size in Bacteria

We carefully selected a compendium of large-scale studies of gene essentiality across a wide range of phylogenetic groups and conditions (Table 1). Our curation process (see methods for details) resulted in 17 *in vitro* experiments for 16 organisms from 6 distinct phyla (Table

1). The datasets used here have important technical and biological differences. The three organisms (*Streptococcus sanguinis*, *E. coli* and *B. subtilis*) showing the lowest numbers of essential genes (Figure 1; Table 1) were evaluated by single-gene knockouts, which is considered the gold standard approach. On the other hand, *Methanococcus maripaludis* (an Archaea) and *Mycobacterium tuberculosis* showed the largest essential gene datasets (Figure 1; Table 1). Both species grow in the presence of CO_2 and have complex nutritional requirements and more genes are required for growth due to suboptimal growth media. However, as shown throughout the manuscript, these datasets are unbiased and can be used in systematic analyses like that reported here.

We found that while genome sizes vary from 475 (*Mycoplasma genitalium*) up to 5,727 (*Burkholderia pseudomallei*) protein-coding genes, the number of essential genes varies from 218 (*S. sanguinis*) to 774 (*M. tuberculosis*) (Figure 1; Table 1), indicating a lack of correlation between the number of essential genes and genome size in Bacteria (Figure 1; Table 1). Essential gene sets are apparently more constrained in prokaryotes than in eukaryotes, in which the fraction of essential genes is more proportional to genome size. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have 1,100-1,300 genes that are essential for growth [29, 30]. Even though the gene complements of these fungi have sizes comparable to larger bacterial genomes, their essential gene sets are more than ~1.4 larger than the largest prokaryotic essential gene set studied here (i.e. *Mycobacterium tuberculosis*, with 774 essential genes). Although systematic screenings are yet unavailable for multicellular eukaryotes, ~3,000 genes have been demonstrably essential for viable development in mouse [31]. We will be able to have a better picture of this phenomenon when more systematic surveys become available for Archaea and eukaryotes.

Genes involved in cellular proliferation are enriched in essential genes

Most studies report genes indispensable for bacteria in rich medium, allowing growth without several biosynthetic pathways. Because these experiments are stress-free, essential genes mainly comprise the basic cellular machinery (e.g. DNA replication and protein translation genes). This observation is supported by the over-representation of essential genes in the *Translation, ribosomal structure and biogenesis* category (J) in all *in vitro* screenings (Figure 2). Our results also show other over-representation patterns in these gene sets (Figure 2). Because experimental determination is largely independent of evolutionary concepts and computational predictions, pathways conserved in few species can also be detected as functionally enriched. For example, *Cell wall, membrane and envelope biogenesis* (M) genes were enriched in many datasets (Figure 2) in spite of the distinct cell wall composition and biosynthetic pathways encoded in gram-positive and gram-negative bacterial genomes [32].

We also analyzed individual essential genes, pathways and their phyletic patterns. *Lipid transport and metabolism* (I) holds relevant differences between Bacteria and Archaea. Isoprenoids (or terpenoids) are important elements of prokaryotic membrane and cell wall [33]. Phospholipids are other critical components of membranes and their biosynthesis is widely conserved in bacteria [34]. Phosphatidate cytidylyltransferase (EC 2.7.7.41) and CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (EC 2.7.8.5), essential enzymes involved in glycerophospholipid metabolism (KEGG: ec00564), were conserved in nearly all bacteria (Table S1). On the other hand, the role of fatty acids in Archaea remains controversial, as the archaeal membrane depends on isoprenoids synthesized by the mevalonate pathway (KEGG: M00095) [35]. The mevalonate pathway comprises the first steps (from acetyl-CoA to isopentenyl-PP) in terpenoid backbone biosynthesis (KEGG: ec00900) in Archaea, fungi and metazoans, while Bacteria and Apicomplexa perform these steps up to isopentenyl-PP through the methylerythritol 4-phosphate pathway (KEGG: M00096) [36]. Enzymes from the mevalonate pathway (i.e. MMP1212, MMP1211, MMP0087 and MMP1335) are essential in *M. maripaludis*, as well as those from the methylerythritol 4-phosphate pathway are essential in Bacteria. Mevalonate pathway genes are essential in *S. sanguinis* (SSA_0338, SSA_0337, SSA_0333, SSA_0335, SSA_0334), as previously shown for *Streptococcus pneumoniae* [37]. Unlike most bacteria, *S. sanguinis* uses the mevalonate pathway, which was horizontally transferred from archaeal or eukaryotic cells [33, 37] and probably replaced the methylerythritol 4-phosphate pathway. Finally, the undecaprenyl pyrophosphate synthase (EC 2.5.1.31), a lipid carrier for peptidoglycan synthesis in bacteria (probably a glycosyl carrier in Archaea) and involved in the final step of terpenoid backbone biosynthesis (ec00900), is essential in archaea and most bacteria, except for mycoplasmas, which do not have cell wall.

Cell cycle control, cell division, chromosome partitioning (D) and *Coenzyme transport and metabolism* (H) were enriched in 58% and 82% of experiments, respectively. Cell division is directly related to cellular mass increase, cytoplasm and DNA partitioning between daughter cells and membrane remodeling. Defective cell division genes may result in asymmetrically-sized daughters [38] or impaired cell division [39]. Many studies on bacterial cell division focus on FtsZ [39], a protein conserved in most bacteria and in Euryarchaeota [40], a phylum that includes *M. maripaludis* and presents a bacterial-type division mechanism [41]. FtsZ is important for septum formation [42] and is essential in all species except *Porphyromonas gingivalis* and *M. maripaludis* (Table S1). The *P. gingivalis* FtsZ gene had 2 insertions in both technical replicates and was not considered essential (Brian Klein, personal communication). *M. maripaludis* has two FtsZ genes (MMP1436 and MMP1500) with identical domain architectures, probably providing a genetic backup to each other.

Coenzyme transport and metabolism (H) comprises pathways whose products are critical for various other pathways. Genes required for the synthesis of coenzyme A (CoA) and biotin (coenzyme R/vitamin H), critical coenzymes in fatty acid oxidation and other metabolic pathways, are required in most species (Table S1). Further, genes involved in the production of nicotinamide (vitamin B3), riboflavin (vitamin B2), folate (vitamin B9) and S-adenosylmethionine are also essential. Nevertheless, genes that are poorly characterized or have no COG annotation account for 28-54% of the essential genes sets. Hence, other functional trends are likely to emerge as genome annotations improve.

Conservation, gene families and the composition of essential gene complements

To investigate the conservation of the essential gene sets, we mapped all protein-coding genes from the 16 prokaryotes studied here to the eggNOG database [43]. Genes assigned to the same non-supervised orthologous group (NOG) were considered homologs.

Conservation across 2,031 genomes available in eggNOG was assessed using the Persistence Index [44] (see methods for details). To avoid biases from the phylogenetic composition of the database, essential gene properties were compared with their non-essential counterparts in the same genome. Strikingly, all essential datasets determined *in vitro* comprise genes that are far more conserved than the non-essential genes (Figure 3). These results support the existence of a highly-conserved core of genes responsible for growth in a common condition (i.e. rich medium), in spite of the wide evolutionary range and other phenomena that affect gene retention/loss (e.g. non-orthologous gene displacement [45]). These observations generalize concepts developed using *E. coli* and *B. subtilis* [21, 44] to the other major bacterial groups and Archaea, implying that higher conservation of essential genes is a common feature in prokaryotes. Interestingly, this trend is apparently attenuated *in vivo* (Figure 3), likely as a consequence of divergent survival strategies to grow under unstable nutrient offer and attacks from the immune system. It is important to bear in mind that *in vivo* screenings typically do not reach saturation and have *in vitro* steps before inoculation, resulting in a potentially underappreciated gene set. However, due to their medical relevance, the lower conservation of genes required *in vivo* deserves further investigation, as discussed below.

In order to evaluate the diversity of the essential gene repertories, we performed a Multiple Correspondence Analysis (MCA) [46], a multivariate method to reduce data dimension and identify systematic patterns of variations in categorical data. We defined the categorical data as the presence/absence of essential genes from each gene set in each NOG. Besides clustering closely-related organisms, MCA allowed us to capture an influence of the environment, reflected by departures from the phylogeny-driven clustering (Figure 4). The three *in vivo* required gene complements from the distantly related *M. tuberculosis* and *F. tularensis novicida* were closely positioned to each other, suggesting that common strategies might be employed when in contact with the immune system (Figure 4). Although there are limitations in these *in vivo* datasets (discussed above), we investigated this important trend in further detail. NOGs that are exclusively essential in *M. tuberculosis in vivo* conditions (i.e. those in which there is no other essential gene in any of the other datasets) were retrieved, aiming to find genes related to infection and pathogenesis (Table S2). This set comprises several lipid metabolism genes, which is coherent with previous reports emphasizing the energetic roles of lipids in *M. tuberculosis in vivo* [15, 47, 48]. Transporters from the MFS and ABC superfamilies and the tetR helix-turn-helix transcriptional repressor Rv3050c were also found as essential in this gene set. Since members of the tetR family have been related to multiple biological processes related with stress [49], Rv3050c might be a critical regulator during *M. tuberculosis* infection. It is also clear that *Burkholderia pseudomallei*, the causative agent of melioidosis, has important peculiarities concerning its essential gene complement (Figure 4) and we analyzed its exclusively essential NOGs, as explained above (Table S2). Strikingly, there are 105 NOGs from which members are essential only in *B. pseudomallei*, including many ABC transporters and metabolic enzymes, supporting its complex metabolic landscape. This gene set also contains transcriptional regulators from the GntR, LysR and AraC families [50, 51], which might be related to the extraordinary antibiotic resistance of *B. pseudomallei* and its capacity to occupy a wide range of niches, from soil to intracellular environments [52]. Importantly, these results cannot be solely explained by *B. pseudomallei* genome size or plasticity [9, 53]. *Burkholderia thailandensis*, which diverged from *B. pseudomallei* around 47 million years ago [9] and also harbors a large and highly plastic genome, has its essential gene set closely grouped to other proteobacteria (Figure 4). Given the scarce treatment options and the classification of *B. pseudomallei* as a potential bioterrorism threat by the U.S. Centers for Disease Control and Prevention, this gene set constitute a valuable source of candidates for downstream experiments to validate their potential as drug or vaccine candidates.

Next, we compared essential genes from the archaea *M. maripaludis* to COG/NOG annotations from the 15 bacterial genomes with *in vitro* essential datasets available. Essential archaeal COG/NOGs with no orthologs in bacteria were considered *M. maripaludis* exclusive essential gene groups, while COG/NOGs with at least one bacterial ortholog were considered shared gene groups. Out of the 520 genes essential in rich medium, 10 were assigned to two COG/NOGs and not considered for further analysis; 194 genes were archaeal exclusive (including 26 genes with no COG/NOG annotation) and 316 genes had bacterial homologs. The eggNOG categories *Replication, recombination and repair* (L) and *Poorly characterized* (S and R) prevailed among exclusive essential genes (Figure S1). Interestingly, three large and almost entirely essential operons (6-8 genes) involved in methane metabolism were identified. Tungstein-containing formylmethanofuran dehydrogenase (DOOR operonID 95836, Table S3) catalyzes the dehydrogenation of formylmethanofuran to methanofuran and CO (EC: 1.2.99.5). This enzymatic complex, present in methanogenic and sulfate-reducing archaea, is related to the first steps in methanogenesis, responsible for CO_2 reduction to methane and autotrophic CO_2 fixation, being crucial for archaeal metabolism [54]. Tetrahydromethanopterin S-methyltransferase and Methyl-coenzyme M reductase operons (DOOR operonIDs 95906 and 95905, respectively) are related to *Coenzyme transport and metabolism*. The former catalyzes the formation of methyl-coenzyme M and tetrahydromethanopterin from coenzyme M and methyltetrahydromethanopterin (EC: 2.1.1.86) [55], whereas the latter plays a role in the final step of methane biosynthesis, reducing methyl-coenzyme M and coenzyme B to methane (EC: 2.8.4.1) [56]. Large-scale methanogen production is of great biotechnological interest and understanding which genes and operons are involved in this process is critical. Since methanogenic Archaea are extremely important in anaerobic decomposition of sewage, optimized methane-producing cells could be used in industrial waste management and as a methane-renewable energy source.

We also sought to identify universal essential genes, as this set may help us to understand some features of the LUCA. We found 19 of such genes in all 16 organisms tested *in vitro* (Table 2), mostly belonging to *Information storage and processing* (J). This gene set comprises 6 aminoacyl-tRNA synthetases, 8 ribosomal proteins and the alpha subunit of DNA polymerase. Other major proteins are secY, the main transmembrane subunit of type II secretion system (Intracellular trafficking, secretion, and vesicular transport, U), and prs, which converts ribose 5-phosphate into phosphoribosyl pyrophosphate (EC: 2.7.6.1), which are essential for purine metabolism. Taken together, these observations are in agreement with the status of protein translation and DNA replication as central biological processes in all organisms. Nevertheless, some groups have long been reported non-orthologous or distantly related genes performing the same functions even in the core machinery between Bacteria and Archaea [45, 57, 58], explaining the small number of universal genes, especially when only experimentally-determined essential genes are considered. Our findings demonstrate the existence of an important core of experimentally determined essential genes shared by Bacteria and an Archaea, which could have been essential in the LUCA as well. Availability of other archaeal essential gene sets will certainly help to evaluate this hypothesis.

Multigene families may be of great adaptive value in the evolution of novel functions and providing biochemical backups [59-61]. We found a strong correlation between the number of genes from multigene families and number of CDSs in the species analyzed here (see methods for details) (Figure 5A; Table S4); together with the lack of correlation between the number of essential genes and CDSs (Figure 1), this result suggests that homologs often compensate single gene loss. We tested this hypothesis and found a strong negative association between the presence of homologs in the genome and essentiality *in vitro* (13/17 with $P < 10^{-5}$; Figure 5B). In other words, under controlled conditions essential genes tend to come from monogenic families. Surprisingly, when we performed the same analysis on conditions closer to the organism lifestyle, such as growth *in vivo* or in macrophages, there is an apparent reversion of this trend with the recruitment of genes from multigene families to essential roles (Figure 5B). A clearer picture is likely to emerge when saturated *in vivo* screenings become available. One may argue that the enrichment of essential genes in monogenic families derive from a technical limitation in single-gene deletion/disruption screening when a homologous backup is available. Nevertheless, the theoretical foundations of gene essentiality lie on a single-gene framework, which is biologically relevant and has proven extremely successful over the past two decades; therefore, our observations have implications for bacterial evolution, as discussed below.

Finally, we have also tested the prominence of horizontal gene transfers (HGTs) in shaping prokaryotic essential gene sets by performing all the analyses described in this section after excluding all genes predicted to have high probability of an HGT event (see methods for details). Very low numbers of essential HGT genes (less than 2%) were found in all species (Table S5), as expected from previous reports [27, 62]; the removal of HGT genes did not affect the statistical significance of our results (data not shown).

In vitro and *in vivo* essential gene sets are extremely dissimilar

The identification of genes required *in vivo* is of great interest, not only in biomedical research, but also from an evolutionary perspective, as these genes are likely to be required in nature. *In vivo* screenings are mostly based on mutant fitness -- when a mutant fails to grow or shows reduced counting in the output pool, it is inferred that the disrupted gene is important for the infection and survival of the pathogen inside the host [15, 17, 47]. Among the selected studies, unique gene sets are found to be required *in vivo* and essential *in vitro* (Figure S2). As discussed above, this overlap might be underappreciated because the mutants are passed *in vitro* before inoculation and many important genes *in vitro* are likely to be critical *in vivo* as well. Nevertheless, genes required *in vivo* but not *in vitro* are functionally diverse (Figure S2), being important candidates for drug intervention.

Over disease progression, mutants must colonize, disseminate and persist under unstable nutrient supply and continuous attacks from the immune system. Accordingly, many genes required *in vivo* are related to metabolism categories (Figure S2). Interestingly, the most predominant categories within the *in vivo* required gene sets are poorly characterized or have no COG assignment (Figure S2). As these genes may play important roles in pathogenesis, we analyzed their protein domain architectures. Some genes required for *F. tularensis novicida* during mice infection are related to coenzyme pyrrolo-quinoline-quinone (PQQ) biosynthesis (FTN_0933, Pfam: PF05402), variable adherence-associated antigen adhesins (FTN_1133, Pfam: PF01540) and DNA repair (FTN_1196, Pfam: PF02575) (Table S6). Further, genes involved in fatty acid synthesis (Rv0100, Pfam: PF00550) and processome of tRNAs or rRNAs (Rv0207c, Pfam: PF01936) are essential in *M. tuberculosis in vivo* conditions and categorized as poorly characterized (Table S6). These results illustrate an open field to phenotypic/functional genomic studies that could shed light on the roles of those genes in complex host-pathogen interactions.

Essential genes are not uniformly distributed inside operons

It has been demonstrated that essential genes are enriched in operons in *E. coli* [26] and we tested whether this is a general feature in other Bacteria and in Archaea. There is a clear trend for essential genes to occupy operons across all 16 prokaryotic genomes ($P \leq 0.05$; Fisher's exact test) (Table S7). Further, the statistical significance is very high in 13 of these conditions ($P < 1.6\times10^{-4}$; Fisher's exact test) (Table S7). Importantly, these results were largely supported even without considering genes encoding ribosomal proteins, which are clustered in large, widely-conserved operons (Table S8). The tendency of essential genes to be in operons was further corroborated using simulated datasets (data not shown). Thus, the prevalence of essential genes in operons is an ancient, high-level prokaryotic feature, probably present in the LUCA. Alternatively, the adaptive value of arranging essential genes in operons is so high that it might have evolved independently in Bacteria and Archaea. Further, we analyzed the association between gene order and essentiality. Gene order is generally preserved in closely related organisms but rapidly decreases with phylogenetic distance [6], except for a few widely-conserved operons [63-65]. However, even such extremely conserved operons are found in distinct arrangements across bacterial and archaeal genomes [63, 66]. Remarkably, we observed that essential genes preferentially occupy the first position in operons containing at least one essential gene (Table 3). Two- and three-sized operons account for the majority (52.3-74.9%) of operons [25, 67, 68], regardless of the presence of essential genes in their structures. Thus, we performed a chi-square test in these 2- and 3-sized operons and confirmed the enrichment of essential genes in the first operon positions in most species, including *M. maripaludis* ($P < 0.01$; Table S9). This scenario is in agreement with a previous observation that essential genes are biased towards the 5′-end half of operons, while pseudogenes tend to be in the 3′-end half of *Mycobacterium leprae* operons [69].

Prokaryotes have ~50% of their genes present in operons [70] and we found that essential genes are enriched in operons in several species (Table S7). Probably due to their higher expression [20], essential genes are more conserved than nonessential genes in terms of phyletic patterns (Figure 3) and sequence similarity [19]. Moreover, essential genes tend to be hubs and form cliques (complete sub-graphs) with each other in protein interaction networks [71, 72]. These features may contribute to the propensity of essential genes to become coordinately expressed with other genes (essential or otherwise) required under similar conditions. Operons reduce the amount of regulatory information needed for optimized transcription of co-regulated genes [27] and under complex regulatory requirements, operons are more likely to evolve than independent promoters in distinct genes [27]. This observation is supported by the more complex regulatory regions of operons when compared to monocistronic genes [27].

Genes from an operon are typically expressed according to their position and a strong correlation between operon length, order and expression has been proposed [73, 74]. Further, these genes generally display decaying expression in a staircase-like manner, with proximal genes (5′) being more expressed than 3′ genes [73, 74]. Based on their codon adaptation index and microarray data [75], essential genes are known to be highly expressed [20, 75]. Thus, the presence of essential genes in the first position in operons (Table 3; Table S9) has direct implications in their higher expression levels [73]. Further, the presence of upstream essential genes in operons increases their chances of being expressed if a mutation hampers the transcription of downstream genes. Taken together the results presented here and elsewhere, we hypothesize that the regulatory regions of 5’-essential genes may drive the regulation and, ultimately, the evolution of whole operons.

Here we reported a systematic analysis of experimentally determined essential genes. We found that essential genes are typically monogenic and more conserved than their nonessential counterparts across thousands of genomes. Extreme gene retention rates are at the foundations of gene persistence, which has been related to gene essentiality and genome organization [44]. Persistence was also extremely useful in the identification of truly essential genes that are not detected in controlled stress-free conditions (e.g. DNA repair genes) [44]. Nevertheless, we showed that many genes essential for growth *in vivo* are not widely conserved and often recruited from multigenic families, suggesting the existence of different survival strategies that co-evolved with the respective bacterial hosts. We propose novel targets for therapeutic or vaccine intervention by exploiting the phyletic patterns of such genes. Moreover, we showed that essential genes are not only preferentially located in operons, but tend to occupy the first position therein, supporting the importance of their regulatory regions in driving the expression of operons. Importantly, many features of gene essentiality in Bacteria are also present in the extremophile archaea *M. maripaludis*, suggesting that there are high order prokaryotic features that could have been either present in the LUCA or evolved independently in the two prokaryotic domains. We believe that the development of synthetic bacterial genomes for biotechnological applications may seriously benefit from an integrated computational and experimental approach based on the many features of essential and persistent genes reported here and elsewhere [27, 44], including not only the essential gene content, but also their specific positioning in operons.

MATERIALS AND METHODS

Data sources

Gene sets were selected after careful assessment of their original publications, using the following criteria for *in vitro* studies: 1) Essentiality must be supported by experimental evidence; 2) the experimental approach must have been systematic, covering at least 80% of the genome when single-gene deletions were used; 3) when transposon mutagenesis was employed, the screening should have reached saturation or near saturation. Only protein-coding genes were analyzed. The only studies that did not have strictly met the criteria above was with *Salmonella enteric*a typhimurium SL1344 [76], which was based on cell fitness reduction when a gene was disrupted by a transposon and; *Bacillus subtilis* [77], for which essential genes were identified with a single crossover recombination technique complemented by a predicted set of essential genes. The former study was included because 95% of required gene set overlaps the essential gene set from a previous study [11], whereas the latter was considered because only 4% (185/4100) of the genes were used for essentiality prediction. A total of 16 organisms (1 Archaea and 15 Bacteria) and 17 *in vitro* screenings were selected (Table 1). Genomes were retrieved from Genbank [78], along with their gene identifiers, coordinates, gene names, strand information and protein sequence, which were extracted from Genbank files. Operon predictions were downloaded from DOOR2 [79], which was reported as one of the most accurate operon prediction repositories [80]. Simulations and data processing were conducted using *in-house* Perl, R (www.r-project.org) and shell scripts (available upon request).

Homology analysis

Homology data were obtained from the eggNOG database v4.0 [43]. Protein sequences were mapped to eggNOG (2,031 core-periphery species) using BLAST [81]. NOGs are an extension of the manually curated COGs [22, 43]. Since the species considered here are already part of the eggNOG database or have close relatives therein, we used strict BLAST criteria, e-value ≤ 10^{-10} and $S \geq 60\%$, where S is the coverage of the shortest sequence (either query or hit). The persistence index of a NOG was computed as previously described [44] as the fraction of the species with at least one homolog in that NOG. The presence/absence patterns of essential genes from a given species in a NOG in each condition were used to create a Boolean matrix that was used to perform a MCA using the FactoMineR package [46]. Functional category enrichment analyses were calculated using the Fisher's exact test ($P < 0.05$). Two genes from the same species were considered part of a multigene family if they share the same NOG. Genes associated with potential HGT events for all species (except *S. enterica* typhimurium SL1344 and *B. fragilis* 638R) were identified with the DarkHorse database [82], which is based on a statistical analysis of archaeal and bacterial genomes for the identification of phylogenetically atypical proteins [82]. Domain architectures were computed using HMMer v3 (E-value ≤ 0.01) [83] and the Pfam 27.0 database [84].

Genome organization

Presence of essential genes in operons—For each dataset, all protein-coding genes were used to build a 2×2 contingency table with the following categories: polycistronic essential genes; polycistronic nonessential genes; monocistronic essential genes and; monocistronic nonessential genes. Statistical associations were evaluated using the Fisher's exact test ($P \leq 0.05$). In addition, the same analysis was performed in 10,000 simulated genomes with the same number of essential genes, operons and genes in each operon. Statistical analyses were performed in R.

Position of essential genes in operons—Only operons with at least one essential gene were considered. As most operons have 2 or 3 genes, we analyzed those for the preferential location of essential genes at the first position using chi-squared tests ($P \leq 0.01$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LUCA	Last Universal Common Ancestor
NOG	non-supervised orthologous group
MCA	Multiple Correspondence Analysis
COG	Clusters of Orthologous Group

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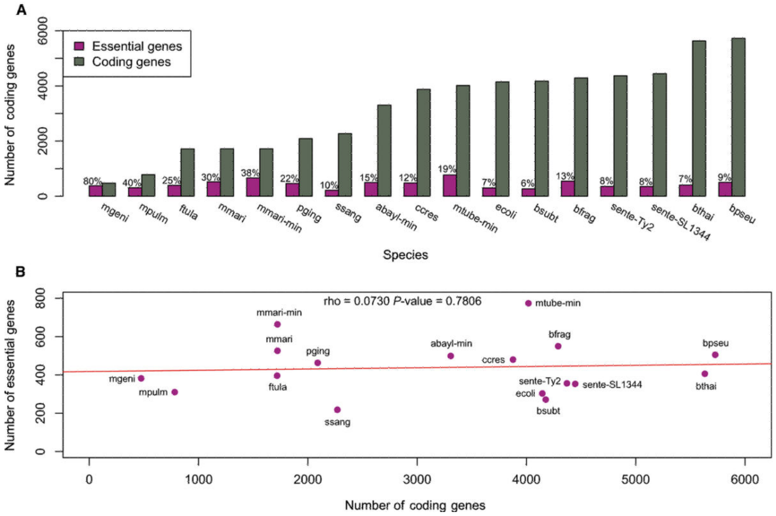
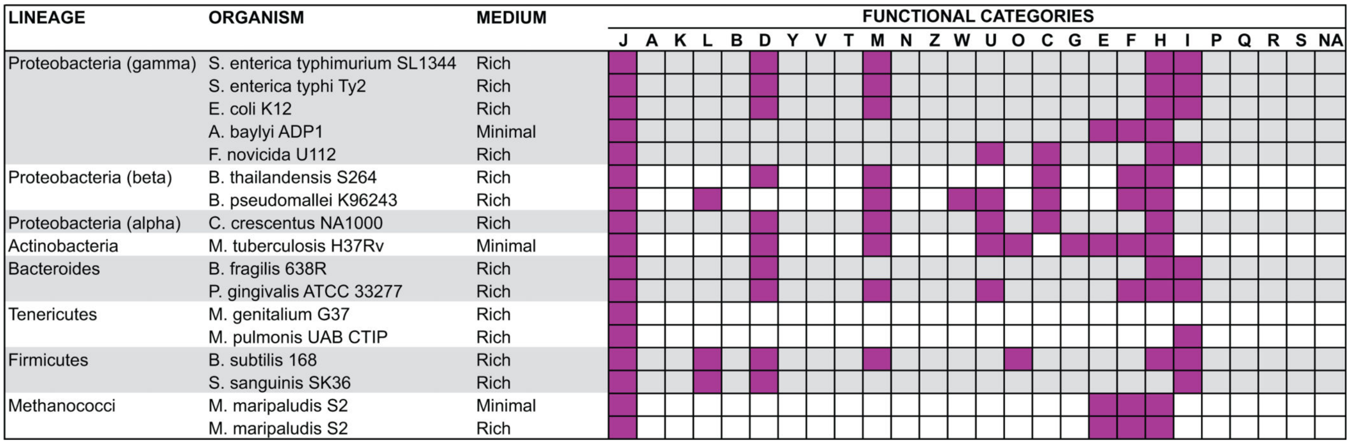


Figure 1. Essential genes obtained from 17 dispensability experiments and their correlation to the total gene complement

A) Percentage of essential protein-coding genes; **B)** Correlation between essential gene set size and genome size. Abbreviations: abayl-min (*Acinetobacter baylyi* ADP1, minimal medium); bfrag (*Bacteroides fragilis* 638R); bpseu (*Burkholderia pseudomallei* K96243); bsubt (*Bacillus subtilis* 168); bthai (*Burkholderia thailandensis* E264); ccres (*Caulobacter crescentus* NA1000); ecoli (*Escherichia coli* K-12); ftula (*Francisella tularensis novicida* U112); mgeni (*Mycoplasma genitalium* G37); mmari (*Methanococcus maripaludis* S2, rich medium); mmarimin (*Methanococcus maripaludis* S2, minimal medium); mpulm (*Mycoplasma pulmonis* CT); mtube-min (*Mycobacterium tuberculosis* H37Rv, minimal medium); pging (*Porphyromonas gingivalis* ATCC 33277); sente-SL1344 (*Salmonella enterica* typhimurium SL1344); sente-Ty2 (*Salmonella enterica* typhi Ty2); ssang (*Streptococcus sanguinis* SK36, minimal medium).



LINEAGE	ORGANISM	MEDIUM	J	A	K	L	B	D	Y	V	T	M	N	Z	W	U	O	C	G	E	F	H	I	P	Q	R	S	NA
Proteobacteria (gamma)	S. enterica typhimurium SL1344	Rich	■					■				■										■	■					
	S. enterica typhi Ty2	Rich	■					■				■										■	■					
	E. coli K12	Rich	■					■				■										■	■					
	A. baylyi ADP1	Minimal	■																	■	■	■						
	F. novicida U112	Rich	■													■		■				■	■					
Proteobacteria (beta)	B. thailandensis S264	Rich	■					■				■						■			■	■						
	B. pseudomallei K96243	Rich	■			■						■			■	■		■			■	■						
Proteobacteria (alpha)	C. crescentus NA1000	Rich	■					■				■				■		■				■						
Actinobacteria	M. tuberculosis H37Rv	Minimal	■					■				■				■	■		■	■	■	■						
Bacteroides	B. fragilis 638R	Rich	■					■														■	■					
	P. gingivalis ATCC 33277	Rich	■					■				■				■					■	■	■					
Tenericutes	M. genitalium G37	Rich	■																									
	M. pulmonis UAB CTIP	Rich	■																				■					
Firmicutes	B. subtilis 168	Rich	■			■		■				■					■					■	■					
	S. sanguinis SK36	Rich	■			■		■															■					
Methanococci	M. maripaludis S2	Minimal	■																	■	■	■						
	M. maripaludis S2	Rich	■																	■	■	■						

Figure 2. Functional categories enriched in essential gene datasets

Squares in magenta represent functional categories enriched in the respective essential gene set (Fisher's exact test; $P < 0.05$).

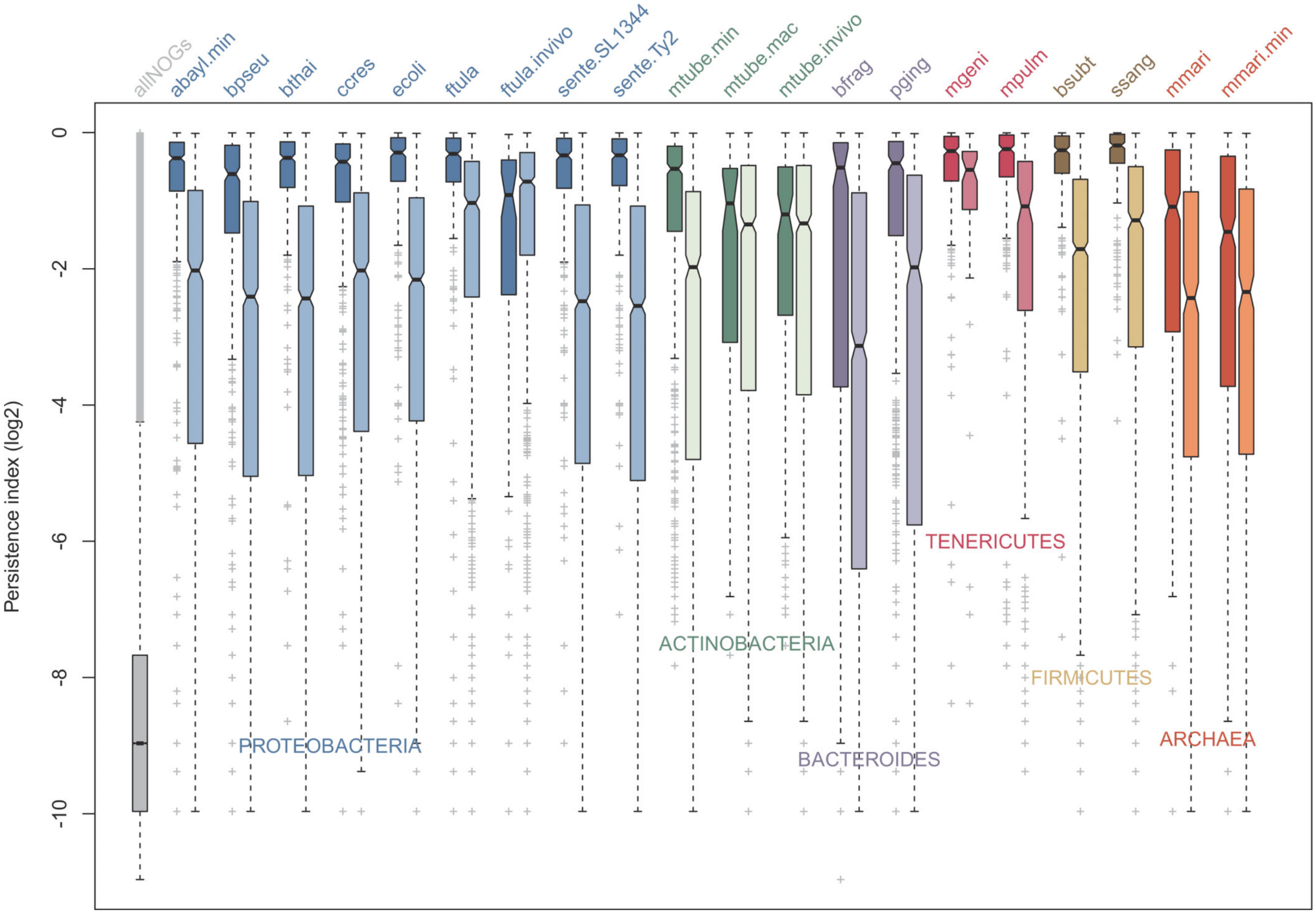


Figure 3. Conservation of essential and nonessential gene sets across thousands of species

Boxplot representation of essential gene sets across thousands of species available in the eggNOG database (see methods for details). Unless indicated otherwise, rich media were used in the screenings. For abbreviations of *in vitro* experiments refer to Figure 1. For *in vivo* experiments: ftula-invivo (*F. tularensis novicida* U112, *in vivo*); mtube-invivo (*Mycobacterium tuberculosis* H37Rv, *in vivo*); and mtube-mac (*Mycobacterium tuberculosis* H37Rv, macrophages). Essential and nonessential gene sets for each condition are side-by-side, in dark and light colors. Proteobacteria, Actinobacteria, Bacteroides, Tenericutes, Firmicutes and Archaea are represented in blue, green, purple, magenta, brown and red, respectively.

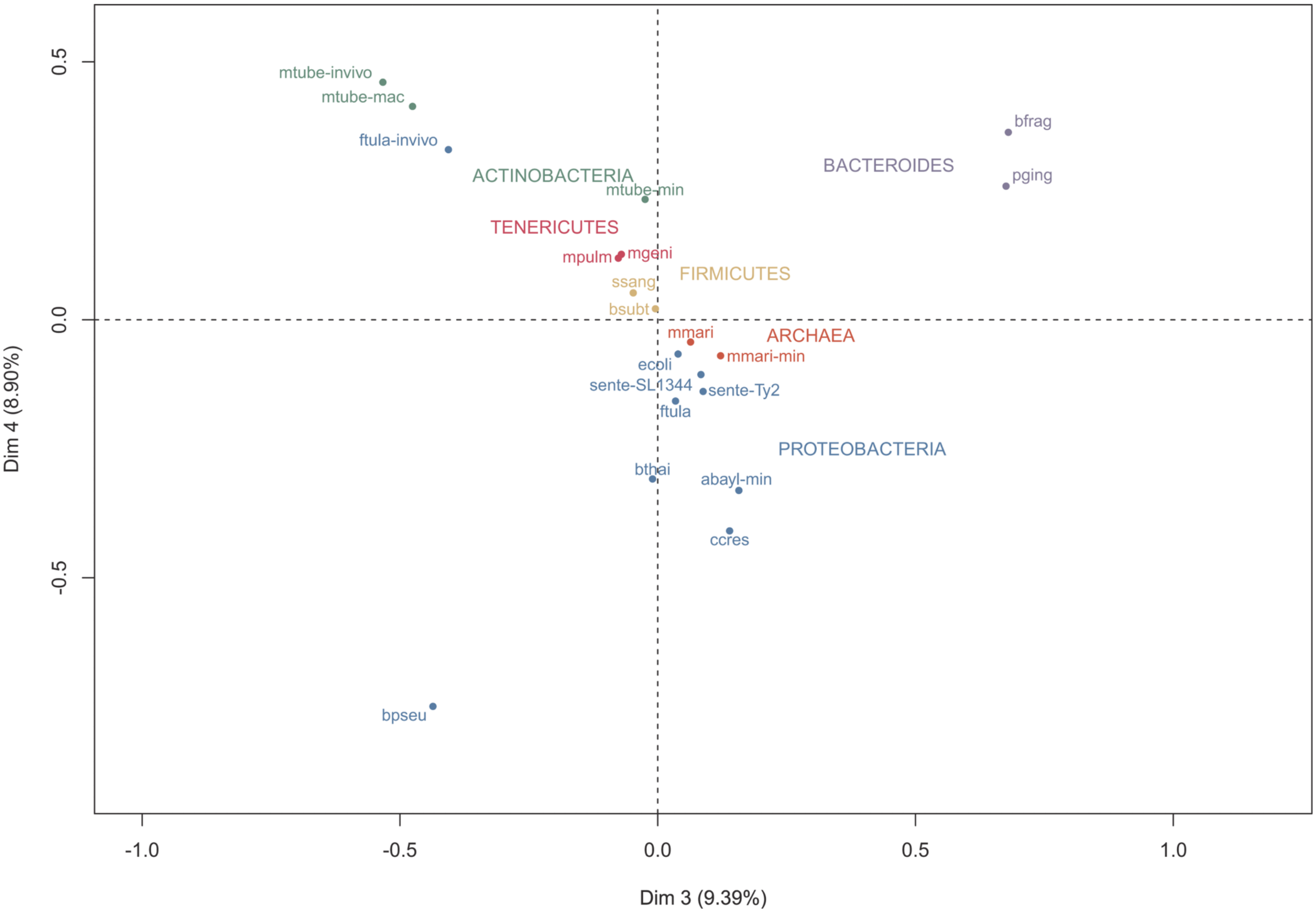


Figure 4. Multiple Correspondence Analysis (MCA) of the presence/absence of essential genes in NOGs

MCA analysis of essential gene sets based on the presence/absence profiles of each mapped NOG. The first two dimensions obtained in MCA were dominated by one or two samples and therefore, are not very useful for separation purposes. Dimensions 3 and 4 allowed an evolutionarily coherent clustering, while still accounting for a significant amount of variance. For abbreviations of *in vitro* experiments refer to Figure 1. For *in vivo* experiments: ftula-invivo (*F. tularensis novicida* U112, *in vivo*); mtube-invivo (*Mycobacterium tuberculosis* H37Rv, *in vivo*); and mtube-mac (*Mycobacterium tuberculosis* H37Rv, macrophages). For color codes, refer to Figure 3.

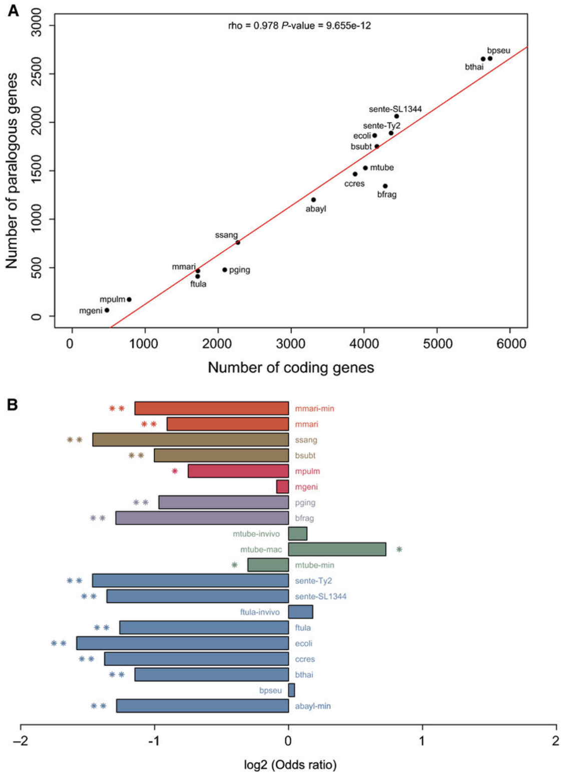


Figure 5. Association between the number of coding genes and gene essentiality with the presence of homologs

A) Total number of coding genes *versus* genes in multigene families: genes with same COG/NOG assignment in a genome were considered part of multigene families. **B)** Gene essentiality *versus* presence of a homolog in the genome: Fisher's exact tests were performed to assess the enrichment of essential genes in multigene families. Bars with one and two asterisks represent P ≤ 10^{-2} and P ≤ 10^{-5}, respectively. For abbreviations of *in vitro* experiments refer to Figure 1. For in vivo experiments: ftula-invivo (*F. tularensis novicida* U112, *in vivo*); mtubeinvivo (*Mycobacterium tuberculosis* H37Rv, *in vivo*); and mtube-mac (*Mycobacterium tuberculosis* H37Rv, macrophages). For color codes, refer to Figure 3.

Table 1

Experimentally-determined essential gene sets used in the present study.

Phylum	Species	Total of essential Genes	Number of CDSs	% of essential genes	Medium	Approach	Ref
Actinobacteria	*Mycobacterium tuberculosis* H37Rv	774	4018	19.20%	Minimal	High-density transposon mutagenesis + Illumina sequencing	[85]
Bacteroides	*Bacteroides fragilis* 638R	550	4290	12.80%	Rich	Transposon delivery vetor + Illumina sequencing	[86]
Bacteroides	*Porphyromonas gingivalis* ATCC 33277	463	2090	22.10%	Rich	Global transposon mutagenesis + Illumina sequencing (TnSeq)	[87]
Firmicutes	*Bacillus subtilis* 168	271	4176	6.40%	Rich	Gene-by-gene inactivation	[77]
Firmicutes	*Streptococcus sanguinis* SK36	218	2270	9.60%	Rich	Systematic gene replacement	[10]
Methanococci	*Methanococcus maripaludis* S2	526	1722	30.50%	Rich	Saturation mutagenesis technique + Illumina sequencing (TnSeq)	[28]
Methanococci	*Methanococcus maripaludis* S2	664	1722	38.50%	Minimal	Saturation mutagenesis technique + Illumina sequencing (TnSeq)	[28]
Proteobacteria (alpha)	*Caulobacter crescentus* NA1000	480	3877	12.40%	Rich	Hyper-saturated transposon mutagenesis + Illumina sequencing	[88]
Proteobacteria (beta)	*Burkholderia pseudomallei* K96243	505	5727	8.80%	Rich	Transposon directed insertion sequencing site (TRADIS)	[9]
Proteobacteria (beta)	*Burkholderia thailandensis* E264	406	5632	7.20%	Rich	Saturation level transposon mutagenesis + Illumina sequencing (TnSeq)	[89]
Proteobacteria (gamma)	*Acinetobacter baylyi* ADP1	499	3307	15.10%	Minimal	Single-gene-deletion	[90]
Proteobacteria (gamma)	*Escherichia coli* K12	303	4145	7.30%	Rich	In-frame single gene deletions	[91]
Proteobacteria (gamma)	*Francisella tularensis novicida* U112	396	1719	23.00%	Rich	Sequence-defined transposon mutant library + Sanger sequencing	[92]
Proteobacteria (gamma)	*Salmonella enterica typhi* Ty2	356	4370	8.10%	Rich	Transposon directed insertion sequencing site (TRADIS)	[76]
Proteobacteria (gamma)	*Salmonella enterica typhimurium* SL1344	353	4446	7.90%	Rich	Transposon directed insertion sequencing site (TRADIS)	[76]
Tenericutes	*Mycoplasma genitalium* G37	382	475	80.40%	Rich	Global transposon mutagenesis + Sanger sequencing	[93]
Tenericutes	*Mycoplasma pulmonis* CT	310	782	39.60%	Rich	Global transposon mutagenesis + Sanger sequencing	[94]

Table 2

Universally conserved essential COG/NOGs

Only essential genes experimentally determined were considered.

INFORMATION STORAGE AND PROCESSING		
Translation, ribosomal structure and biogenesis (J)	COG0018	Arginyl-tRNA synthetase
	COG0008	Glutamyl- and glutaminyl-tRNA synthetases
	COG0124	Histidyl-tRNA synthetase
	COG0495	Leucyl-tRNA synthetase
	COG0442	Prolyl-tRNA synthetase
	COG0172	Seryl-tRNA synthetase
	COG0090	Ribosomal protein L2
	COG0087	Ribosomal protein L3
	COG0088	Ribosomal protein L4
	COG0097	Ribosomal protein L6P/L9E
	COG0102	Ribosomal protein L13
	COG0092	Ribosomal protein S3
	COG0522	Ribosomal protein S4 and related proteins
	COG0098	Ribosomal protein S5
Transcription (K)	COG0202	DNA-directed RNA polymerase, alpha subunit/40 kD subunit
Replication, recombination and repair (L)	COG0592	DNA polymerase III sliding clamp (beta) subunit, PCNA homolog

CELLULAR PROCESSES AND SIGNALING		
Cell cycle control, cell division, chromosome partitioning (D)	COG0037	Predicted ATPase of the PP-loop superfamily implicated in cell cycle control
Intracellular trafficking, secretion, and vesicular transport (U)	COG0201	Preprotein translocase subunit SecY
	COG0552	Signal recognition particle GTPase (protein FtsY) **

METABOLISM		
Nucleotide transport and metabolism (F)	COG0462	Phosphoribosylpyrophosphate synthetase

* When considering only the *Methanococcus maripaludis* minimal medium;

Table 3

Relationship between essential genes and operon position.

Species	Medium	Position in the operon													Ess-op[a]	Ess-genes[b]	Operons[c]
		1	2	3	4	5	6	7	8	9	10	11	13	14			
A. baylyi ADP1	Minimal	120 (65.2%)	50	7	3	3	1	0	0	0	0	0	0	0	184	348	643
B. subtilis 168	Rich	59 (58.4%)	33	7	2	0	0	0	0	0	0	0	0	0	101	206	818
B. fragilis 638R	Rich	155 (69.1%)	42	19	4	1	1	2	0	0	0	0	0	0	224	407	956
B. pseudomallei K96243	Rich	126 (55.2%)	58	21	13	6	3	0	0	0	0	0	0	1	228	403	1146
B. thailandensis S264	Rich	97 (57.4%)	47	13	8	2	1	1	0	0	0	0	0	0	169	310	1155
C. crescentus NA1000	Rich	123 (67.2%)	38	14	3	5	0	0	0	0	0	0	0	0	183	338	844
E. coli K-12	Rich	67 (52.8%)	38	7	8	5	0	1	0	0	0	1	0	0	127	232	851
F. tularensis novicida U112	Rich	94 (59.9%)	42	12	7	2	0	0	0	0	0	0	0	0	157	321	373
M. maripaludis S2	Rich	102 (62.2%)	44	13	3	1	1	0	0	0	0	0	0	0	164	351	362
M. maripaludis S2	Minimal	128 (62.7%)	56	12	5	3	0	0	0	0	0	0	0	0	204	423	362
M. tuberculosis H37Rv	Minimal	193 (59.2%)	92	24	10	4	1	0	2	0	0	0	0	0	326	582	895
M. genitalium G37	Rich	69 (82.1%)	12	1	2	0	0	0	0	0	0	0	0	0	84	343	89
M. pulmonis UAB	Rich	81 (74.3%)	21	5	2	0	0	0	0	0	0	0	0	0	109	247	175
P. gingivalis ATCC	Rich	144 (83.7%)	16	6	3	3	0	0	0	0	0	0	0	0	172	385	455
S. enterica typhimurium SL1344	Rich	75 (57.7%)	37	10	3	3	2	0	0	0	0	0	0	0	130	238	881
S. enterica typhi Ty2	Rich	76 (54.7%)	44	7	8	2	0	0	1	0	1	0	0	0	139	264	838
S. sanguinis SK36	Rich	50 (57.5%)	27	6	2	1	1	0	0	0	0	0	0	0	87	159	489

[a] Number of operons with at least one essential gene

[b] Number of polycistronic essential genes

[c] Total number of operons.