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# Protein Localization Analysis of Essential Genes in Prokaryotes

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Essential genes, those critical for the survival of an organism under certain conditions, play a significant role in pharmaceuticals and synthetic biology. Knowledge of protein localization is invaluable for understanding their function as well as the interaction of different proteins. However, systematical examination of essential genes from the aspect of the localizations of proteins they encode has not been explored before. Here, a comprehensive protein localization analysis of essential genes in 27 prokaryotes including 24 bacteria, 2 mycoplasmas and 1 archaeon has been performed. Both statistical analysis of localization information in these genomes and GO (Gene Ontology) terms enriched in the essential genes show that proteins encoded by essential genes are enriched in internal location sites, while exist in cell envelope with a lower proportion compared with non-essential ones. Meanwhile, there are few essential proteins in the external subcellular location sites such as flagellum and fimbrium, and proteins encoded by non-essential genes tend to have diverse localizations. These results would provide further insights into the understanding of fundamental functions needed to support a cellular life and improve gene essentiality prediction by taking the protein localization and enriched GO terms into consideration.

Regardless of the immense differences between bacterial genomes in their size and gene repertoires, all the genomes must contain enough information giving the cell the ability to maintain metabolic homeostasis, reproduction, and evolution, the three basic properties of cellular life<sup>1</sup>. Among all the genes in an organism, what genes are indispensable to fulfill these functions? To address this problem, a concept of essential gene was proposed. Essential genes are those indispensable for the survival of an organism under certain conditions, and the functions they encode are therefore considered a foundation of life<sup>2–4</sup>. Investigation of essential genes is becoming an increasingly appealing issue not only because it will shed new light on the understanding of life at its simplest level, but also because it has much significance in practical use such as pharmaceuticals and synthetic biology<sup>5–7</sup>.

An intuitive way to identify an essential gene is to detect whether the inactivation of this gene is lethal. Previous approaches used to identify essential genes include global transposon mutagenesis strategies, inhibition of gene expression using antisense RNA and systematic gene inactivation of each individual gene present in a genome<sup>2,8</sup>. More recently, high-throughput sequencing has been applied together with high-density transposon-mediated mutagenesis, which has increased the number of prokaryotic species involved in gene essentiality research dramatically<sup>9</sup>. In the last few years, great progresses not only *in vivo* but also *in silico* have been made. For example, bacterial essential genes have been showed more evolutionarily conserved than non-essential ones and tend to reside in the leading strand<sup>10,11</sup>. Based on these progresses, gene essentiality prediction models and tools have also been developed<sup>12–15</sup>.

Our study is focused on the protein location of essential genes. In general case, proteins must be transported to the appropriate location to perform their designated function. The location sites in prokaryotic cells can be reduced to three groups: internal structures, cell envelope and external structures. The uppermost internal structure is cytoplasm, a jelly-like substance where all proteins are synthesized and most of them remain<sup>16,17</sup>. The main structures found in the cytoplasm are the ribosomes and one (or a few) chromosome (s) which are essential to the functions of all prokaryotic cells. The cell envelope is composed of cytoplasmic membrane and cell wall in Gram-positive bacteria. While in Gram-negative bacteria, the cell envelope location sites include the cytoplasmic membrane, the outer membrane and the periplasm, which is the space between the two membranes. Most external structures such as flagella, fimbriae, capsule, and slime layer are specific structures that are found in some, but not all bacteria<sup>18</sup>.



Knowledge of protein localization is invaluable for understanding their function as well as the interaction of different proteins<sup>19</sup>. When other information is not available, the subcellular localization will also be helpful in the annotation for new proteins. In the medical microbiology, subcellular location knowledge can help identify therapeutic intervention points rapidly during the drug discovery progress. For example, because of their localization, secreted proteins and membrane proteins are easily accessible by drug molecules<sup>20</sup>. Because of the critical functions of essential genes, it was hypothesized that proteins encoded by essential genes are enriched in internal location sites, while exist in cell envelope with a lower proportion compared with non-essential ones. In the current study, some analyses were performed to test this hypothesis.

## Results and discussion

We selected 27 prokaryotic organisms to analyze the protein location of the essential and non-essential genes. The data used in the current study are obtained from DEG (a database of essential genes, available at <http://www.essentialgene.org/>)<sup>21</sup> and are displayed in Table 1. To elucidate the evolutionary relationship among the organisms, the phylogenetic tree was constructed. The lines at the top of Figure 1 are the phylogenetic tree of the organisms used in the current study. The tree was constructed using the MEGA6 program (Statistical Method: Maximum Likelihood, Test of Phylogeny: Bootstrap method, No. of Bootstrap Replications: 1000)<sup>22</sup> with the sequences of 16S ribosomal RNA of the 27 organisms downloaded from the NCBI FTP site (<ftp://ftp.ncbi.nih.gov/genomes/Bacteria>). Based on the clades of the tree, the organisms can be divided into 4 groups: 20 Gram-negative bacteria (pink clades in Figure 1), 4 Gram-positive bacteria (purple clades), 2 mycoplasmas (blue clades) and 1 archaeon (green clades). To which group *Mycobacterium tuberculosis* H37Rv should be classified is disputable. We treated it as Gram-negative bacterium due to its cell structure.

**Protein localizations are different between essential and non-essential genes.** We first submitted the amino acid sequences of both essential and non-essential genes in the 27 organisms to PSORTb and obtained the protein localization records. With precision values >97% for both archaea and bacteria, PSORTb 3.0 is the most precise prokaryotic localization prediction tool available. Compared with other localization prediction tools, PSORTb is able to discriminate between Gram-positive and Gram-negative bacteria, which makes it a more suitable tool for the current study<sup>23</sup>. The final prediction includes five Gram-negative localization sites (cytoplasm, cytoplasmic membrane, periplasm, outer membrane and extracellular space) and four Gram-positive localization sites (cytoplasm, cytoplasmic membrane, cell wall and extracellular space)<sup>24</sup>. When a protein might have multiple localization sites, PSORTb will output the most possible localization site. Then we calculated the proportions of proteins located in the location sites for essential and non-essential genes respectively. The results are displayed in Figure 2. The average percentages of proteins located in cytoplasm are 64.40% and 43.88% for essential and non-essential genes, respectively. The Student's *t* test shows that the difference is statistically significant ( $p=1.57 \times 10^{-10}$ ). For all the organisms except *Vibrio cholerae* N16961, the percentages of proteins located in cytoplasm for essential genes are higher than those for non-essential genes (Figure 2a). The reason for the anomalous outcome in *Vibrio cholerae* N16961 may be the higher proportion of 'unknown' predicted results (43.13%) compared with the average percentage (16.43%). To test this hypothesis, we used another tool CELLO<sup>25,26</sup> to predict the protein localization in *Vibrio cholerae* N16961 again. Then the percentages become 71.08% and 54.31% for essential and non-essential genes respectively, which is in accordance with the results of the other 26 organisms. These results suggest that proteins encoded by essential genes are

enriched in cytoplasm. The average percentages of proteins located in cytoplasm membrane are 16.73% and 23.35% for essential and non-essential genes, respectively. The Student's *t* test shows that the difference is statistically significant ( $p=1.33 \times 10^{-5}$ ). The bars in Figure 2a shows that in 23 (85.19%) of the 27 groups of data, the percentages of proteins located in cytoplasm membrane for non-essential genes are higher than those for essential genes. For both essential and non-essential proteins, the proportions of secreted proteins are quite low, just 0.50% essential proteins and 1.54% non-essential proteins are located in extracellular space. With Student's *t* test  $p=1.95 \times 10^{-4}$ , it's credible that the proportion of non-essential proteins located in extracellular is significantly higher than that of essential ones (Figure 2a).

Cytoplasm, cytoplasm membrane and extracellular are protein location sites involved in all the 4 groups of organisms. Figure 2a shows percentages of proteins located in the three location sites of all the genomes. In Figure 2b, the 3 location sites mentioned are not ubiquitous. Just the 4 Gram-positive bacteria and 1 archaeon have the cell wall structure. Outer membrane and periplasm are the unique structure of Gram-negative bacteria. Figure 2b presents the average percentages of proteins located in the three location sites for essential and non-essential genes in the related genomes. The proportions of non-essential proteins located in periplasm, outer membrane and cell wall are higher than those of essential proteins. The corresponding *p* values are  $4.06 \times 10^{-6}$ ,  $3.06 \times 10^{-3}$  and  $4.68 \times 10^{-2}$ . All the values are less than 0.05, which means that the differences are statistically significant. Since cytoplasm membrane, cell wall, periplasm and outer membrane together form cell envelope, we could reach the conclusion that proteins encoded by essential genes exist in cell envelope with a lower proportion compared with non-essential ones. We found that protein localization differences between essential and non-essential genes are more significant in Gram-positive bacteria than those in Gram-negative bacteria, which may be due to the simple cell structures in Gram-positive bacteria.

Other factors that may influence the protein localization differences, such as the multiple localization of a protein, the reliability of protein localization prediction and the source of non-essential genes, are also discussed here. On average, 2.47% of the essential proteins and 2.70% of the non-essential proteins in the prediction of PSORTb have been annotated with multiple localization sites (the percentages of multiple localization proteins in each dataset are listed in Table 1). Therefore, the issue of multiple localization of a protein only bring a very slight impact on the accuracy of the statistical results due to the low percentages. Since the prediction result might not be perfectly precise, some experimental data were also employed. The protein localization information was obtained from the Universal Protein Resource (UniProt; <http://www.uniprot.org>)<sup>27</sup>. Captured from literatures, the data in UniProt is credible. We selected *Bacillus subtilis* 168, *Escherichia coli* MG1655 and *Mycoplasma genitalium* G37 as model genomes for Gram-positive bacteria, Gram-negative bacteria and mycoplasmas respectively, due to their higher percentages of the proteins with localization information. On average, 47.03% of the essential genes and 44.91% of the non-essential genes in these genomes have annotated localization information. We defined "unknown" as subcellular location for the proteins without annotated localization information. Among the proteins with localization information, 3.54% of the essential proteins and 3.35% of the non-essential proteins have multiple localization sites, which is close to the statistical result obtained from the prediction of PSORTb. Since multiple localization protein can locate in any site mentioned in its annotation, all the related site groups counted the protein in the calculation here. Figure 3 shows the distribution of essential proteins (the inner ring of the doughnut chart) and non-essential proteins (the outer ring of the doughnut chart) in *B. subtilis* 168, *E. coli* MG1655 and *M. genitalium* G37. In all the three doughnut charts, the percentages of the essential proteins located in cytoplasm are



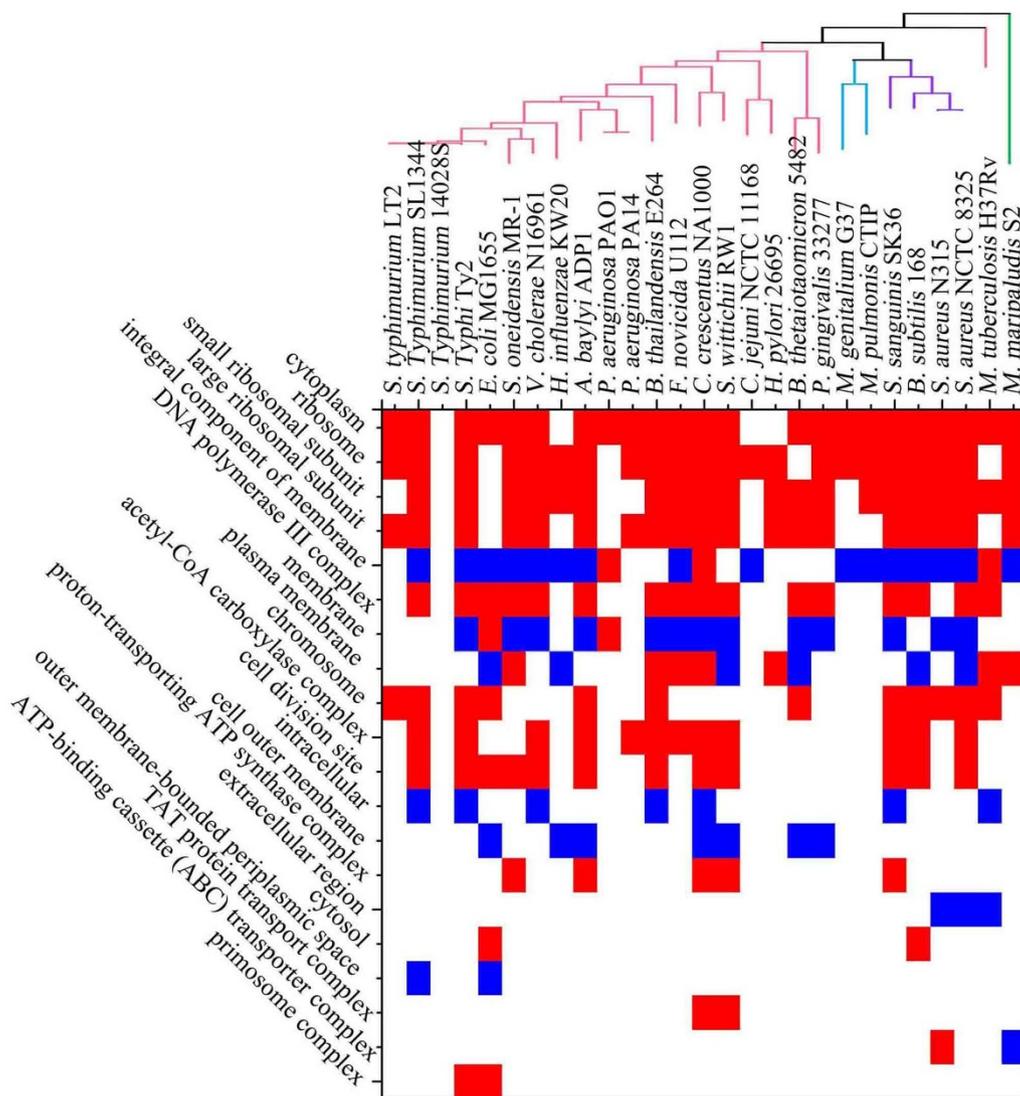
Table 1 | The information of the organisms used in the current study

Organism	Group <sup>a</sup>	RefSeq	Dataset of essential gene <sup>b</sup>	Dataset of non-essential gene <sup>b</sup>	Source <sup>c</sup>
<i>Acinetobacter baylyi</i> ADP1	Bacteria (-)	NC_005966	499, 90.38%, 2.81%	2594, 76.21%, 2.08%	I
<i>Bacillus subtilis</i> 168	Bacteria (+)	NC_000964	271, 94.83%, 1.48%	3955, 81.85%, 1.29%	II
<i>Bacteroides thetaiotaomicron</i> VPI-5482	Bacteria (-)	NC_004663	325, 84.62%, 5.23%	4453, 65.10%, 5.19%	II
<i>Burkholderia thailandensis</i> E264	Bacteria (-)	NC_007650	42, 84.73%, 4.93%	2314, 70.65%, 3.23%	II
	Bacteria (-)	NC_007651	364, 84.73%, 4.93%	2912, 70.65%, 3.23%	II
<i>Campylobacter jejuni</i> NCTC 11168	Bacteria (-)	NC_002163	228, 76.75%, 3.07%	1395, 80.43%, 2.94%	II
<i>Caulobacter crescentus</i> NA1000	Bacteria (-)	NC_011916	480, 83.96%, 4.79%	3224, 63.31%, 3.97%	I
<i>Escherichia coli</i> MG1655	Bacteria (-)	NC_000913	609, 80.13%, 2.79%	2923, 82.18%, 2.39%	I
<i>Francisella novicida</i> U112	Bacteria (-)	NC_008601	392, 87.76%, 3.83%	1329, 76.98%, 3.01%	II
<i>Haemophilus influenzae</i> Rd KW20	Bacteria (-)	NC_000907	642, 87.07%, 1.25%	512, 87.11%, 1.95%	I
<i>Helicobacter pylori</i> 26695	Bacteria (-)	NC_000915	323, 76.47%, 2.48%	1135, 76.83%, 4.58%	I
<i>Methanococcus maripaludis</i> S2	Archaeon	NC_005791	519, 92.49%, 0.39%	1077, 86.72%, 0.09%	I
<i>Mycobacterium tuberculosis</i> H37Rv	Bacteria (-)	NC_000962	687, 85.01%, 3.78%	3070, 61.34%, 2.31%	I
<i>Mycoplasma genitalium</i> G37	Mycoplasmata	NC_000908	381, 78.48%, 0.52%	94, 67.02%, 0.00%	I
<i>Mycoplasma pulmonis</i> UAB CTIP	Mycoplasmata	NC_002771	310, 80.00%, 0.65%	322, 60.25%, 0.62%	I
<i>Paraphromonas gingivalis</i> ATCC 33277	Bacteria (-)	NC_010729	463, 87.26%, 1.94%	1627, 62.81%, 3.01%	II
<i>Pseudomonas aeruginosa</i> PAO1	Bacteria (-)	NC_002516	117, 85.47%, 1.71%	5454, 76.88%, 3.04%	II
<i>Pseudomonas aeruginosa</i> UCBPP-PA14	Bacteria (-)	NC_008463	335, 79.10%, 1.79%	960, 65.83%, 1.98%	I
<i>Salmonella enterica</i> serovar Typhi Ty2	Bacteria (-)	NC_004631	358, 90.78%, 2.79%	3906, 73.35%, 2.18%	I
<i>Salmonella enterica</i> serovar Typhimurium SL1344	Bacteria (-)	NC_016810	353, 88.67%, 2.55%	4035, 74.65%, 2.55%	I
<i>Salmonella enterica</i> serovar Typhimurium 140285	Bacteria (-)	NC_016856	105, 90.48%, 1.90%	5210, 63.88%, 2.13%	II
	Bacteria (-)	NC_003197	230, 87.83%, 3.91%	4228, 74.83%, 2.46%	II
<i>Shewanella oneidensis</i> MR-1	Bacteria (-)	NC_004347	402, 88.81%, 3.48%	1103, 86.49%, 3.45%	I
<i>Sphingomonas wittichii</i> RW1	Bacteria (-)	NC_009511	535, 76.26%, 2.80%	4315, 72.28%, 4.47%	II
<i>Staphylococcus aureus</i> N315	Bacteria (+)	NC_002745	302, 95.03%, 0.66%	2281, 80.45%, 1.18%	II
<i>Staphylococcus aureus</i> NCTC 8325	Bacteria (+)	NC_007795	351, 90.03%, 1.99%	2541, 76.62%, 0.87%	II
<i>Streptococcus sanguinis</i> SK36	Bacteria (+)	NC_009009	218, 94.50%, 0.46%	2052, 82.36%, 1.17%	I
<i>Vibrio cholerae</i> N1 6961	Bacteria (-)	NC_002505	565, 56.87%, 1.41%	2105, 78.05%, 2.89%	II
	Bacteria (-)	NC_002506	214, 56.87%, 1.41%	838, 78.05%, 2.89%	II

<sup>a</sup>Bacteria (+) Gram-positive bacteria; Bacteria (-) Gram-negative bacteria.

<sup>b</sup>The dataset description usually contain three numbers: X, Y%, Z%. X is the number of essential (or non-essential) genes of the organism. Y% is the prediction coverage of essential (or non-essential) genes. Z% is the percentage of proteins which may have multiple localization sites among essential (or non-essential) genes.

<sup>c</sup>Source of the non-essential genes. I, the non-essential genes are obtained based on the original literatures. II, the non-essential genes are the complementary set of essential genes.



**Figure 1** | The plot of statistically significant GO terms in the category of cellular component incorporating the phylogenetic information.

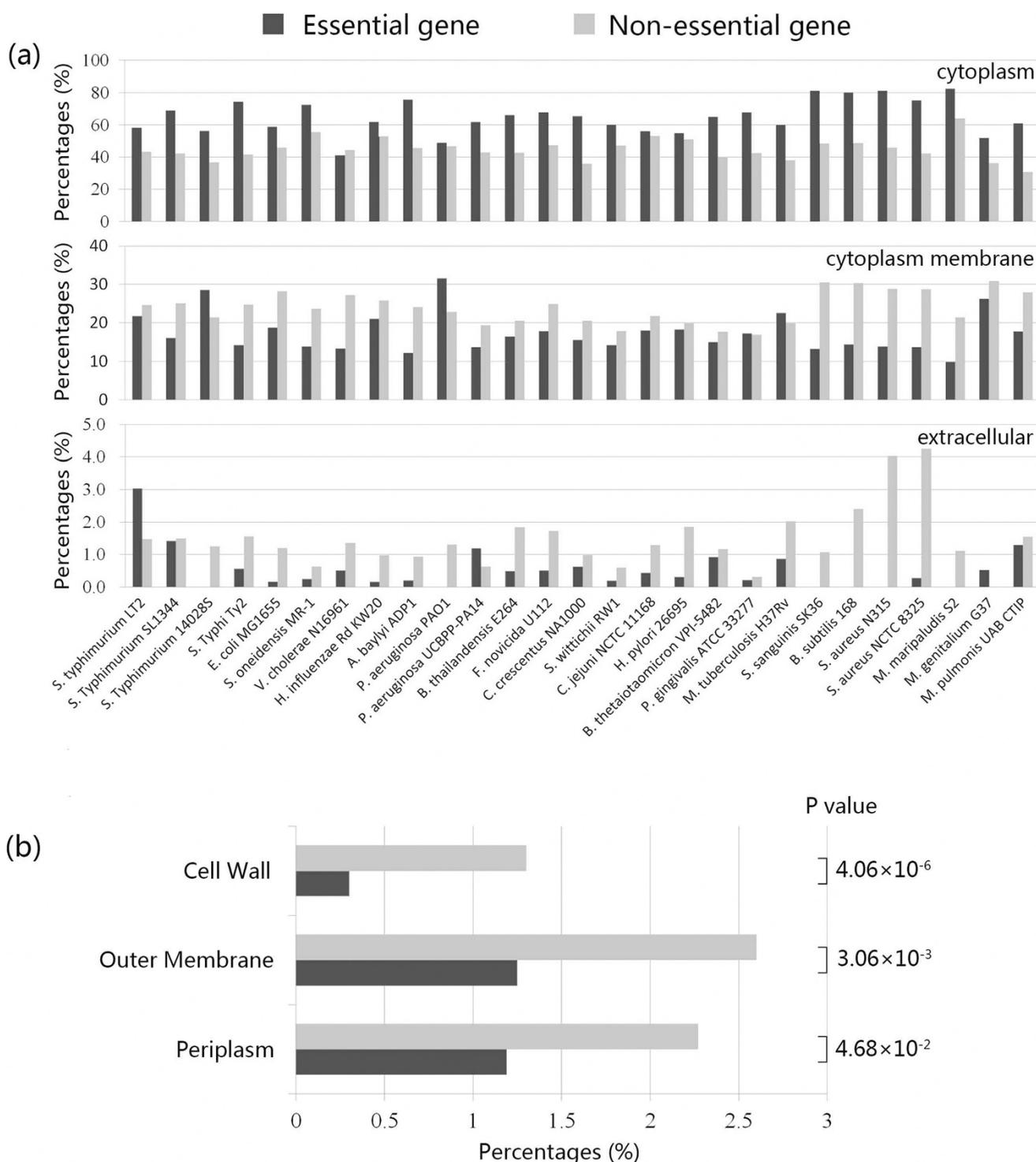
Every GO term with  $p$  value less than 0.05 in over two organisms according to the results of Fisher's exact tests is listed in the vertical axis. If the GO term is over-represented in the organism listed in the horizontal axis, the cell at the crossing of the row and column is red. Blue boxes represent that the GO term is under-represented in the organism of the column. If the GO term is not statistically significant in the organism, the box is white. The lines at the top of the figure are the phylogenetic tree of the organisms used in the current study.

higher than those of non-essential proteins, and the proteins encoded by essential genes exist in cell envelope with a lower proportion compared with non-essential ones. These conclusions are consistent with the prediction results of PSORTb. Comparisons were also made between groups classified according to the source of non-essential genes presented in Table 1. We found the differences are more significant in the organisms whose non-essential genes are obtained based on the original literatures. The reason may be that non-essential from the original literatures are more reliable than those from the complementary set of essential genes.

#### Protein localization analysis of essential genes based on GO terms.

The Gene Ontology (GO) is one of the most useful terms and controlled vocabularies for describing the roles of genes and gene product characteristics. The ontology covers three domains: cellular component, molecular function and biological process. Cellular component refers to the place in the cell where a gene product is active. The molecular function is the elemental activities of a gene product at the molecular level. Biological process is defined as a biological objective to which the gene or gene product contributes<sup>28</sup>.

The Fisher's exact test was employed to obtain the GO terms enriched in the essential genes of 27 prokaryotes.  $P$  values less than 0.05 were considered statistically significant. Figure 1 represents the statistically significant GO terms in the category of cell component. As can be seen from this figure, in this category, GO:0005737 (cytoplasm), GO:0005840 (ribosome) and GO:0015935 (small ribosomal subunit) are the over-represented essential GO terms in all the 4 groups of organisms under analysis. Essential genes are more enriched in cytoplasm, ribosome, small ribosomal subunit and large ribosomal subunit, which are the major cell components for cell functions such as energy metabolism, nucleic acid translation and transcription, whereas GO:0016021 (integral component of membrane), GO:0016020 (membrane), GO:0005886 (plasma membrane), GO:0005622 (intracellular) and GO:0009279 (cell outer membrane) are under-represented in over 6 organisms, which means that proteins in cell components such as membrane have no much relationship with essential genes and are more likely to be encoded by non-essential genes. In addition, the membrane related GO terms are species-specific due to the different structure of cell envelope. For example, besides GO:0009279 (cell outer membrane), GO:0030288



**Figure 2** | (a) Percentages of proteins located in cytoplasm, cytoplasm membrane and extracellular for essential and non-essential genes in the 27 genomes. (b) Average percentages of proteins located in periplasm, outer membrane and cell wall for essential and non-essential genes in the related genomes.

(outer membrane-bounded periplasmic space) and GO:0042597 (periplasmic space) are particularly under-represented in *E. coli* MG1655, which is caused by the complicated cell structure of Gram-negative bacteria. This result can be construed as another evidence to the conclusion that proteins encoded by essential genes tend to locate in cytoplasm.

The Fisher's exact test was also employed to obtain enriched GO terms in the category of biological process and molecular function. GO:0007049 (cell cycle), GO:0006260 (DNA replication),

GO:0009252 (peptidoglycan biosynthetic process), GO:0051301 (cell division), GO:0065002 (intracellular protein transmembrane transport), GO:0006265 (DNA topological change) and GO:0006184 (GTP catabolic process) are the most significantly over-represented biological process GO terms. These process are all indispensable for a cell and take place in cytoplasm or ribosome. The GO terms under-represented in over 6 organisms in this category are GO:0006355 (regulation of transcription, DNA-templated), GO:0035556 (intracellular signal transduction), GO:0006200 (ATP



catabolic process), GO:0005975 (carbohydrate metabolic process) and GO:0055114 (oxidation-reduction process). For the GO terms relating to molecular function, the most significantly over-represented molecular functions are GO:0003735 (structural constituent of ribosome), GO:0019843 (rRNA binding), GO:0005524 (ATP binding), GO:0000049 (tRNA binding), GO:0000287 (magnesium ion binding) and GO:0005525 (GTP binding). While GO:0003700 (sequence-specific DNA binding transcription factor activity), GO:0003677 (DNA binding), GO:0003824 (catalytic activity), GO:0051539 (4 iron, 4 sulfur cluster binding), GO:0000155 (phosphorelay sensor kinase activity), GO:0043565 (sequence-specific DNA binding), GO:0000156 (phosphorelay response regulator activity) and GO:0004872 (receptor activity) are significantly under-represented in more than 6 organisms.

## Conclusion

Our results show the protein localization difference between essential and non-essential genes in prokaryotes. Essential proteins are enriched in cytoplasm. The proportions of non-essential genes locating in cytoplasm membrane, periplasm, outer membrane, cell wall and extracellular are significantly higher than those of essential genes. The Fisher's exact test of GO terms reached a coincident conclusion. Taking the protein localization and protein function into consideration comprehensively, we can know more about essential genes. These results would provide further insights into the understanding of fundamental functions needed to support a cellular life and improve gene essentiality prediction by taking the protein localization and enriched GO terms into consideration.

## Methods

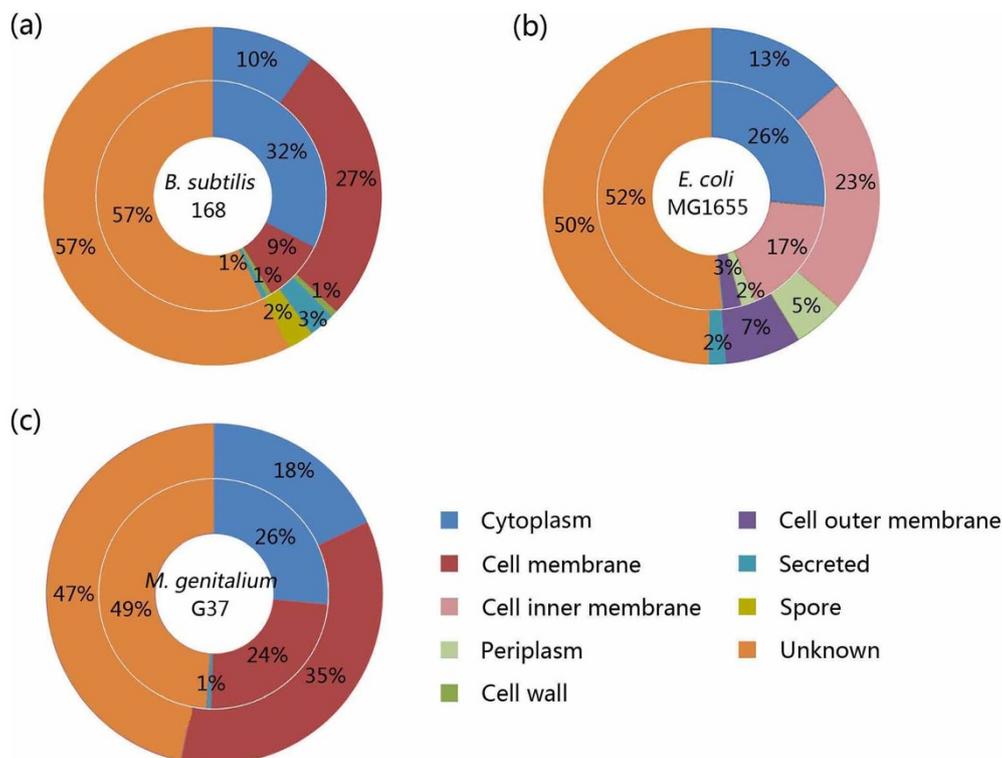
**Bioinformatics Databases.** DEG is a database of essential genes (<http://www.essentialgene.org/>). The newly released DEG 10 has been developed to accommodate the quantitative and qualitative advancements brought by the progressive identification methods. Currently available records of both essential and non-essential genes among a wide range of organisms can be downloaded from DEG 10, making it possible to compare the two different types of genes in many aspects<sup>21</sup>.

27 prokaryotic organisms including 24 bacteria, 2 mycoplasmas and *Methanococcus maripaludis* S2, the only record of the Archaea domain were selected to analyze the protein localization and GO distribution of the essential and non-essential genes. There are 31 bacterial records corresponding to 27 organisms in the database in total and 26 sets of data were selected in the current study. *Streptococcus pneumoniae* was not chosen for the lack of non-essential genes. Since the essential genes were not genome-widely identified, it's not reasonable to regard the complementary set of essential genes as non-essential genes in *Streptococcus pneumoniae*<sup>29,30</sup>. In the case of multiple records for one organism, the one with the most convincing experimental methods was chosen. The non-essential genes in *Methanococcus maripaludis* S2 and 13 bacteria such as *Escherichia coli* MG1655 are obtained based on the original literatures, while non-essential genes in other 12 organisms such as *Bacillus subtilis* 168 are the complementary set of essential genes. The information of the organisms used in the current study are displayed in Table 1.

The three model genomes' subcellular location information and the Gene Ontology (GO) terms used for the analysis in the current study were downloaded from the Universal Protein Resource (UniProt; <http://www.uniprot.org>). Maintained by the UniProt Consortium, UniProt is committed to providing biologists with a comprehensive, high-quality and freely accessible resource of protein sequences and functional annotation<sup>27</sup>. Among the wealth of annotation data, detailed GO annotation statements are included. A comprehensive set of evidenced-based associations between terms from the GO resource and UniProtKB proteins are provided in GO annotation dataset, which is maintained by external collaborating GO Consortium groups<sup>31</sup>.

**Software Tools.** PSORTb is the most precise bacterial localization prediction tool available<sup>24</sup>. PSORTb 3.0 comprises multiple analytical modules, each of them is carried out independently. Every module returns to a prediction either a protein is belonging or not belonging to a particular localization site, or a result of 'unknown' by analyzing one biological feature known to influence subcellular location. All the results are then integrated to generate a final prediction. The likelihood of a protein being at a specific localization site is showed by a score. When a protein might locate in more than one sites, PSORTb will output the most possible localization site. Because PSORTb 3.0 added the capability of predicting subcellular localizations of archaeal proteins, we can obtain the localization information of *Methanococcus maripaludis* S2 with this tool. Compared with other localization prediction tools, PSORTb is able to discriminate between Gram-positive and Gram-negative bacteria, which makes it a more suitable tool for the current study<sup>23</sup>.

CELLO is another localization prediction tool which uses the support vector machines trained by multiple feature vectors based on *n*-peptide compositions<sup>25,26</sup>. We used this tool to predict the protein localizations of *Vibrio cholerae* N16961, for whom the result presented by PSORTb had a high proportion of 'unknown'. The phylogenetic tree was constructed using the MEGA6 program<sup>22</sup>.



**Figure 3** | Distribution of essential proteins (the inner ring of the doughnut chart) and non-essential proteins (the outer ring of the doughnut chart) in (a) *Bacillus subtilis* 168, (b) *Escherichia coli* MG1655 and (c) *Mycoplasma genitalium* G37.



**Test Method.** The Student's *t* test was performed to test the significance of difference between the proportions of proteins located in the sites for essential and non-essential genes. The Student's *t* test is a method of testing whether the means of two groups are statistically different from each other. *P* values less than 0.05 were considered statistically significant.

To obtain the GO terms enriched in the essential genes of 27 prokaryotes, the Fisher's exact test was employed. Fisher's exact test is a statistical significance test used for small sample sizes. The most common use of Fisher's exact test is for  $2 \times 2$  tables, but it is valid for all sample sizes<sup>32</sup>. *P* values less than 0.05 were considered statistically significant.

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## Author contributions

F.G. conceived and designed the study. C.P. performed the data analysis and drafted the manuscript. Both authors edited the manuscript and approved the final manuscript.

## Additional information

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