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Gene context conservation of a higher order than operons

Warren C. Lathe III, Berend Snel and Peer Bork

Operons, co-transcribed and co-regulated contiguous sets of genes, are poorly conserved over short periods of evolutionary time. The gene order, gene content and regulatory mechanisms of operons can be very different, even in closely related species. Here, we present several lines of evidence which suggest that, although an operon and its individual genes and regulatory structures are rearranged when comparing the genomes of different species, this rearrangement is a conservative process. Genomic rearrangements invariably maintain individual genes in very specific functional and regulatory contexts. We call this conserved context an uber-operon.

THE REGULATORY AND neighborhood context (Box 1) of most genes is seen as generally fluid[1,2]. A study of 11 genomes[3] showed that the gene order and content of nearly all the known operons of the *Escherichia coli* and *Bacillus subtilis* genomes are either missing or incomplete in other species, suggesting that genomes are randomly rearranged.

A few genes are known to have highly conserved neighborhoods in many bacterial genomes[4,5]. For example, the *rplC* and *rplD* ribosomal genes are invariably found immediately adjacent to each other in the 22 bacterial genomes completed as of submission of this article. It has been suggested that such highly conserved neighbors are due to the fact that the products of these genes interact physically or functionally[6,7]. And yet, these conserved pairs of genes are the exception rather than the rule and, even when the gene order within an operon is conserved, regulation can be distinct in different species[8]. The regulatory and neighborhood context of most genes varies greatly when comparing genomes of different species.

This apparent fluidity masks a greater conservation of regulatory and neighborhood context. In reality, the different operon architectures seen from genome to genome result from very conservative rearrangements of genes. Examples from three disparate cellular systems (translational machinery, flagellar structure and chemotaxis, and ABC transporter genes) show that, although genomic rearrangements cause variation in the immediate neighborhood of a gene, many genes are maintained over evolutionary time within the context of a discrete set of functionally related genes. We call this set of genes that is conserved at a higher level of organization an uber-operon.

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A translation-associated uber-operon

The exploitation of context information, such as neighborhood, is becoming an important tool in function prediction based on genomic sequence[5–7,9–12]. However, as mentioned above, only a few neighborhood relations and operons are strictly conserved over a wide range of species[4,5]. This is also true for ribosomal operons[13,14], despite the sequence conservation of the individual genes therein. Wachtershauser suggested that the ribosomal gene cluster in the 'universal ancestor' was broken up into smaller clusters during evolution[13]. We propose that the picture is somewhat more complex. Rather than the break up of a large ancestral gene cluster, the evolution of the ribosomal clusters appears to have involved the joining of clusters, break up into smaller clusters and the rearrangement of these into new clusters in a conservative fashion.

The extent of conservative operon reassortment can be illustrated by the neighborhood of one translation-associated gene (*tufA*) that is always found within ribosomal operons. This gene codes for an elongation factor involved in translation and was originally described as part of the *str* operon in *E. coli*[15]. Although the neighborhoods vary for *tufA* in the different genomes, *tufA* invariably occurs together with ribosomal and other translation-associated genes (Fig. 1). It is not only found in the putative ancestral neighborhood[13] of *rpsJ* and *fusA* but also in the neighborhood of different translation-associated genes including *rpmG* (*Helicobacter pylori*), *secE* (*Chlamydia pneumoniae*) and others (*Mycoplasma genitalium*, *Methanococcus jannaschii*). There were no exceptions to a translation-associated neighborhood for *tufA* in any of the 15 genomes studied here.

Phylogenetic analysis of the genes displayed in Fig. 1 does not indicate any detectable lateral transfer of genes across divergent species boundaries[13] (W.C. Lathe, unpublished). From this

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Box 1. Definitions and concepts

Definitions

In this article 'neighbors' and 'neighborhood' mean two genes that are separated by less than 250 bp in the same transcriptional orientation and in which the intervening sequence does not include an open reading frame. These definitions are similar to (but use shorter distances than) those reported by Overbeek *et al.*[7]

Because many operons have not been described experimentally in most species used here, we use the term 'operon' to indicate a putative operon and define it as a cluster of functionally related genes in which each gene within the cluster is separated by less than 250 bp and all genes of the cluster are in the same transcriptional orientation. These neighboring genes and clusters might or might not be co-regulated.

An 'uber-operon' (as described in the text) is discovered and characterized as follows. First, a gene and its orthologs are identified for a chosen number of genomes (15 in this article). Second, the conserved neighbors of these orthologous genes are determined. A gene neighbor is considered to be conserved if it is found as a neighbor to the originally chosen orthologous genes in at least three genomes (in the dataset used, some genomes pairs might be evolutionarily closely related and thus, the fact that genes were neighbors might be due to time and chance and not to conservation). Third, the orthologs of these newly determined conserved neighbors are determined in all the genomes. The conserved neighbors of these additional orthologs are then determined and added to the 'set'.

Steps 1–3 are repeated for several iterations until no new conserved neighbors are found and the number of genes converges to a discrete set (new neighbors found in one genome only are considered random events for this study). This set often converges to a finite number of genes that are functionally related. The neighbor of any single gene from the set will statistically be another gene from within the set. The addition of more genomes to the procedure does not add significantly to the number of genes in the set.

The uber-operon described in this article is thus a discrete set of functionally related genes in which any individual gene from the set is found in the neighborhood of at least one other gene from the set in a significant number (n–1 in most cases) of all studied genomes, suggesting that any rearrangements of gene clusters invariably maintains individual genes within the context of the larger. A preliminary step[25] towards automating this process can be found at http://www.bork.embl-heidelberg.de/STRING/.

Concept

A conceptual scenario of an uber-operon over evolutionary time is shown in Fig. I. In this scenario, the ancestral genome contains three clusters of similarly regulated and functionally related genes, A–H, in three separate genomic locations. As the population diverges and the genome is rearranged, the clusters are rearranged into new clusters. Further evolutionary rearrangements of the genome, instead of breaking up the clusters even further, rearrange the clusters and the individual genes so that they are invariably maintained in the neighborhoods of genes or clusters of the larger set. Rarely, a cluster will be rearranged with a new gene of related function (gene 1 of species III) and newly included into the larger uber-operon set. This would signify a change in the regulation and biochemistry of the species.

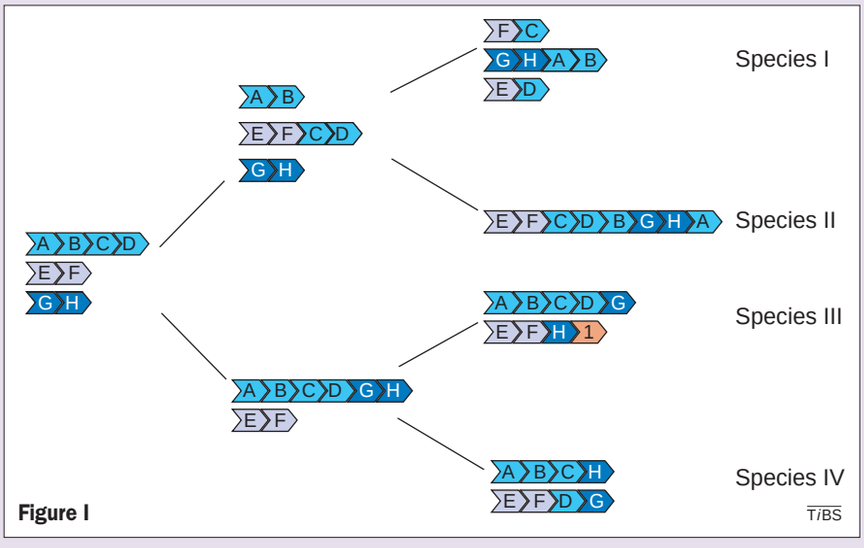

Figure I

Further rearrangements could be postulated. For example, in species IV, the ABCH and the EFDG clusters might later be rearranged to form a new larger cluster of ABCHEFDG. The break up of operons when comparing genomes can thus be shown to be conservative in a larger context. For example, if one were to observe an operon in species III, ABCDG, and then to search for a similar operon in other related species, the picture here would suggest that this 'operon' is not intact in the other genomes (AB only in species I, B alone and CDG rearranged in species II, and missing D in species IV). However, as this scenario shows, the apparent 'break up' of operons is instead a non-random rearrangement of genes so that they are maintained within the same transcriptional and regulatory context.

phylogenetic analysis and from the pattern of gene rearrangements, a likely evolutionary scenario points to an ancient operon containing at least the genes *rpsL*, *rpsG*, *fusA*, *tufA* and *rpsJ* (Fig. 1a). In the Archaea, this ancient operon has probably broken up in several lineages but has been retained in *Methanobacterium thermoautotrophicum*. At the root of the Eubacteria, this operon is also predicted to be rearranged upstream of a gene cluster that is conserved in many genomes (the cluster includes genes orthologous to those from the *E. coli s10*, *spc* and *alpha* operons). Alternatively, this universal ancestor might have included a larger cluster that was broken up in the archaeal lineage.

Although clusters are broken up during evolution, they are often rearranged with new clusters of functionally related genes. Any given evolutionary scenario will require the break up and recombination of various sets of clusters. However, the clusters remain in an 'uber-operon' in which, although the exact neighborhood of each particular gene is not necessarily conserved, the gene is invariably maintained in a transcriptional neighborhood of associated genes from a discrete set (in this case, ribosomal and translation related).

An iterative search of the orthologs of the genes shown in Fig. 1 and their neighbors was carried out to determine whether these genes belonged to a larger set of conservatively rearranged genes[16] (Box 1). The procedure converged on a set of 43 genes in Eubacteria (50 in Archaea owing to fissions of RNA polymerase genes and the addition to the set of several ribosomal genes without orthologs in Eubacteria) that recombine almost exclusively with each other in

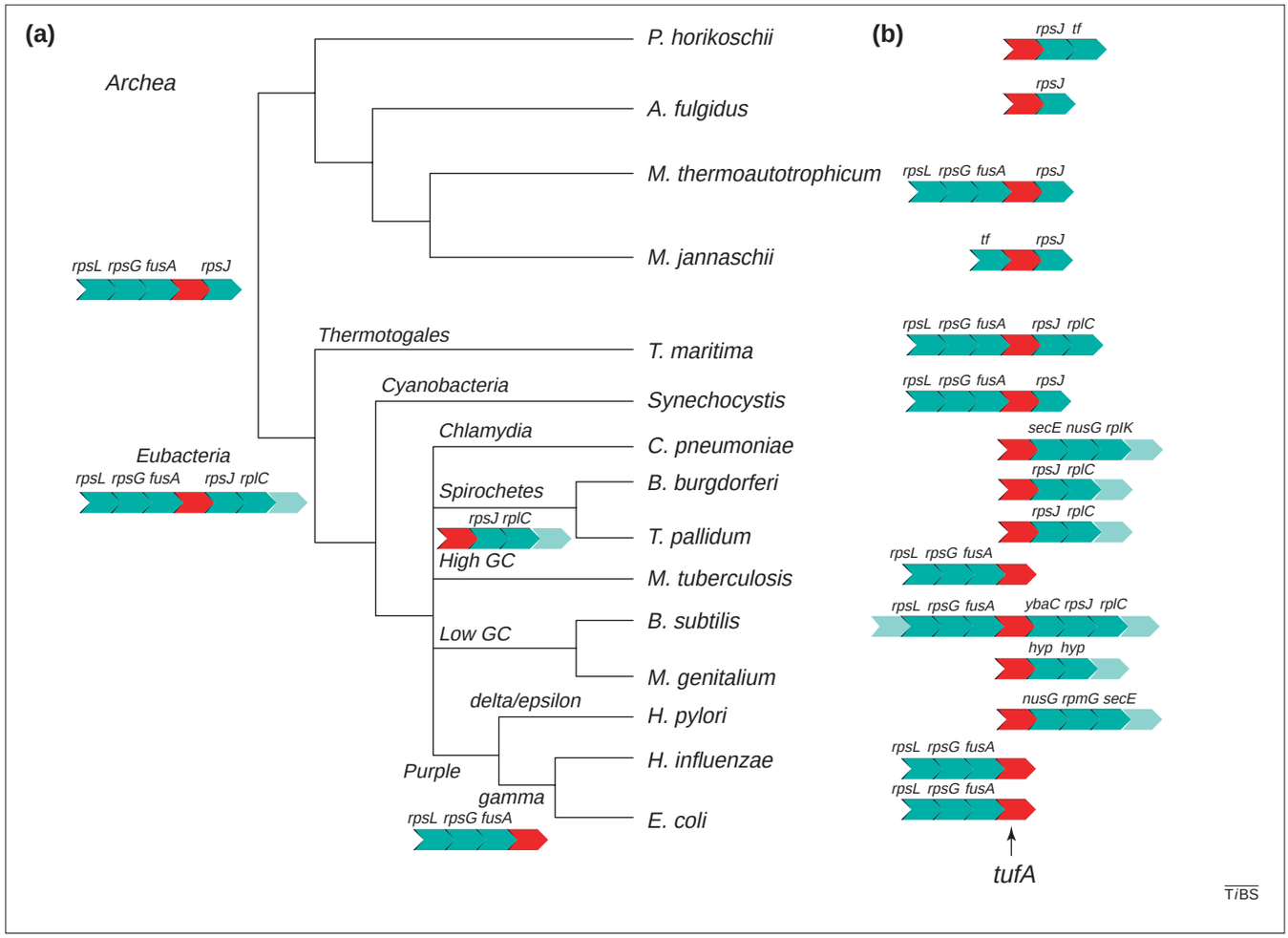

Figure 1

(a) A cladogram based on reported relationships of species[22] included in this article and data obtained from phylogenetic analysis of individual genes [*tufA* (in red), *rpsL*, *rpsG*, *fusA* and *rpsJ*]. Predicted ancestral operons deduced from these trees are indicated at some branches. **(b)** The variable neighborhood of the *tufA* gene (in red) is shown for each species. Light-green boxes indicate further translation-related genes upstream or downstream of the clusters shown. Full descriptions and complete uber-operon set are shown at http://www.bork.embl-heidelberg.de/uberoperon. Genes *rpsL*, *rpsG* and *rpsJ* encode proteins S12, S7 and S10, respectively, of the small ribosomal subunit; *rplC* and *rplK* encode proteins L3 and L11, respectively, of the large ribosomal subunit; *fusA* and *tufA* code for translation elongation factors; *nusG* encodes a transcription antitermination factor involved in regulation of ribosomal gene transcription; and *secE* encodes a subunit for *a* integral membrane protein complex involved in the secretion of newly translated proteins. All sequences and locations for coding and non-coding regions were obtained from the databases referenced at http://www.tigr.org/tdb/. Orthologs were determined and obtained using the protocol reported in Ref. 16. Searches for particular homologous genes were performed using PSI-BLAST (Ref. 17). DNA coding sequences were aligned using ClustalX (Ref. 23) using default parameters (modification of the parameters changed alignments insignificantly). Phylogenies were constructed by maximum parsimony phylogenetic analysis (heuristic search, closest addition) using PAUP (Ref. 24) and was based on full-length amino acid sequences. Trees constructed by maximum likelihood and neighbor joining (using PAUP) gave similar topologies and conclusions.

divergent species and that are located within between one and seven distinct operons, depending on the species. The addition of new genomes does not add to this set. The probability that *tufA* (or any single gene from the uber-operon) will be found as a neighbor of one of the subset of 43 ribosomal genes in all 15 genomes is effectively zero and shows that this is a non-random event (W.C. Lathe and S.R. Sunyaev, unpublished). All of these genes are translation associated and form an uber-operon (a full table of the translation-associated uber-operon and two others that are reported below is at http://www.bork.embl-heidelberg.de/uberoperon).

Possible predictions based on uber-operons

This concept of an uber-operon might assist genome annotation and the discovery of regulatory elements. For example, a few apparent exceptions to the translation-associated uber-operon described above were observed, but closer examination of each case revealed the original genome annotation to be incomplete. In a transcriptional unit downstream of the *rpmG* gene in *M. genitalium*, two genes had originally been annotated as 'hypothetical' [open reading frames (ORFs) MG054 and MG055]. Although *rpmG* is in the neighborhood context of translation-associated uber-operon genes in other genomes, it was apparently isolated from the uber-operon genes in the *M. genitalium* genome. This apparent exception to the uber-operon concept was cause for further investigation of the unannotated ORFs in the neighborhood of *rpmG*. Subsequent PSI-BLAST searches[17] of the downstream hypothetical genes revealed homology with *B. subtilis secE* and *nusG*, two genes that had not yet been annotated in *M. genitalium* but were part of the uber-operon (these ORFs have subsequently been annotated).

Even without known homology, the uber-operon concept might allow function

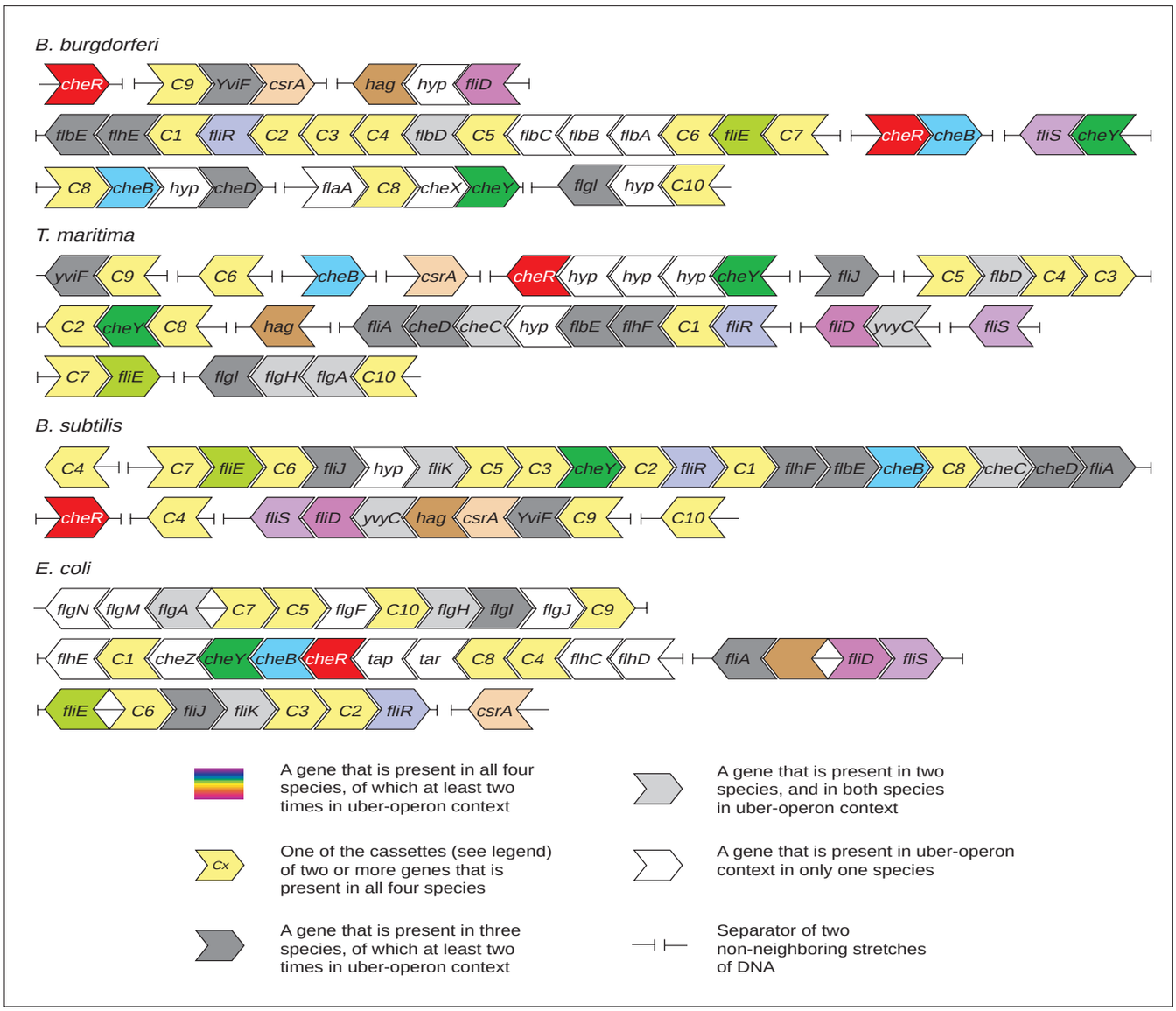

Figure 2

The genomic organization for four species of (i) the genes from the flagellar uber-operon (colored), (ii) genes that occur in the context of the uber-operon but not in all species (gray) and (iii) genes that happen to occur once in the context of the uber-operon (white). The white genes are mostly absent from complete genomes of the other species. The composition of the cassettes is (transcription direction from left to right): C1, (*fliB*, *flhA*); C2, (*fliO*, *fliP*, *fliQ*); C3, (*fliL*, *fliM*, *fliN*); C4, (*motA*, *motB*); C5, (*flgE*, *flgD*); C6, (*fliF*, *fliG*, *fliH*, *fliI*); C7, (*flgB*, *flgC*); C8, (*cheW*, *cheA*); C9, (*flgK*, *flgL*); C10, (*flhO*, *flgG*). The white genes labeled 'hyp' are hypothetical (genes with unknown function) and are not homologous to each other.

predictions using context information. For example, a hypothetical gene in *Pyrococcus horikoschii* directly upstream of the *fusA* elongation factor gene, which is often found adjacent to *tufA* in many other genomes (Fig. 2), has putative orthologs in other genomes that are located in ribosomal operons (e.g. between *rpL37a* and *rplA* in *M. thermoautotrophicum*). Thus, one can predict a translation-associated function because of its apparent inclusion in this uber-operon.

Uber-operons in two other systems of genes

Ribosomal and translation-associated proteins are both essential and ubiquitous, and their neighborhoods are among the most conserved in prokaryotic genomes. To test the universality of the concept of uber-operons, we studied flagellum-related genes (found only in a subset of the 15 species considered) and glutamate ABC transport operons (these genes are known to change substrate specificity readily and so their functionality is poorly conserved). The flagellum system includes structural, chemotaxis-related and other genes necessary for the function of flagella.

Iterative searches for orthologs and conserved neighborhood (as described above) also revealed an uber-operon with only a few exceptions; that is, the presence of genes within the flagella operons of some species that are functionally only loosely associated with the flagellar machinery. For example, the largest flagellar operon, consisting of 26 genes in *Borrelia burgdorferi*, is rearranged in other species with additional flagellum-related genes of a larger set. Figure 2 shows that these rearrangements have the conservative pattern of an uber-operon. The figure is a real-world example of the rearrangements described in Box 1.

To show that there are different levels of conservation and to reduce the amount of presented information, we use cassettes that symbolize two or

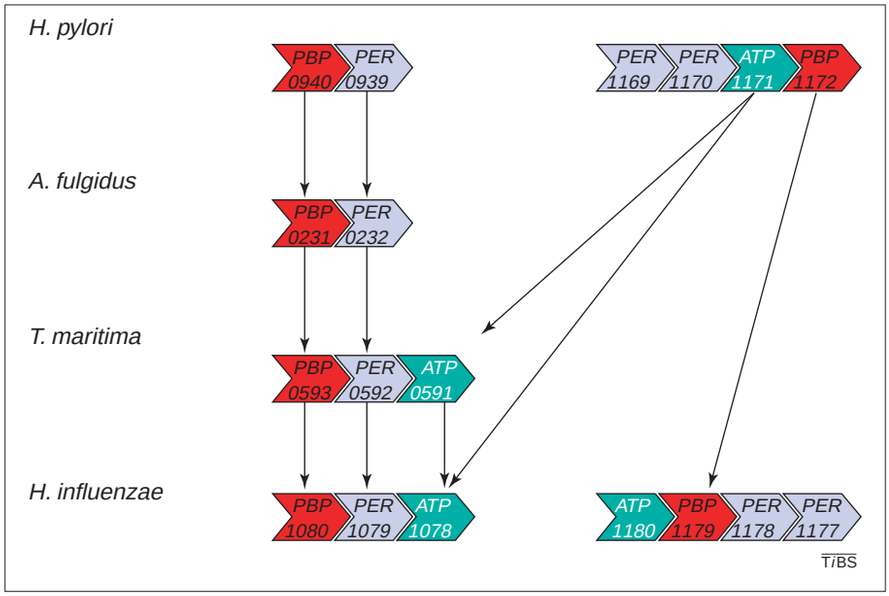

Figure 3

Six ABC transporter operons for amino acid transport. ABC transporter operons usually, but not always, consist of one or two permeases, a periplasmic-binding protein and an ATP-binding protein. Based on orthology[16] and/or annotation, these ABC transporter operons are thought to be involved in glutamine transport with the exception of the first operon shown for *Helicobacter pylori* (HP0940 and HP0939; unspecified amino acid transport) and the second operon shown for *Haemophilus influenzae* (HI1180, HI1179, HI1178 and HI1177; arginine transport). The functions of the proteins in the operons are shown using red for periplasmic-binding proteins (PBP), green for ATP-binding proteins (ATP) and gray for permeases (PER).

more genes that are in the same specific order in all four species. When we look at the neighborhood of cassette 8 (C8) in the four species, we see that in *B. burgdorferi*[18] (in which C8 is duplicated), one copy is located 5′ of *cheB* and the other is located between *flaA* and *cheX*. In *Thermotoga maritima*, C8 lies 5′ of *cheY*; in *B. subtilis*, C8 is located 3′ of *cheB* and 5′ of *cheC*; and, finally, in *E. coli*, C8 is located 3′ of C4 and 5′ of *tar*. All of these genes are flagellum related. Although C8 is not conservatively located between the same two genes, it is located in the context of other flagellum-related genes in the pattern of an uber-operon. Other genes and cassettes of genes of this set are similarly found in these conservative uber-operon rearrangements.

A similar pattern is also detectable in ABC transporter operons (Fig. 3). These contain a much more complex set of genes owing to myriad duplications, deletions, paralogous relationships and alterations of functions between lineages. Delineating an uber-operon in the ABC transporter system is a complex undertaking and any uber-operon can change frequently in evolutionary history. However, several clusters suggest that the uber-operon concept still applies to this system. Arrows between individual genes in Fig. 3 denote orthologs as determined by the method referred to earlier[16]. As shown here, although these ABC transporter operons have different functions within and between species, the individual orthologous genes have been rearranged between these operons to create new ones in an uber-operon fashion. For example, genes from two separate ABC transporter operons in *H. pylori* have been recombined to form a new operon in *T. maritima*.

Thus, the detection of an uber-operon in a system with little functional conservation in evolution indicates the possible general applicability of the concept.

Evolution of uber-operons

Conserved neighboring gene pairs often code for proteins that interact physically[5,6,9,10]. The structure of the ribosomal subunits has recently been determined[19,20], and some of the individual proteins therein that physically interact are encoded by tightly associated and conserved gene pairs. For example, the *rpsG* and *rpsL* genes (encoding small subunit ribsomal proteins S7 and S12, respectively) are invariably found as a gene pair in all prokaryotic genomes. The products of these two genes are the only two that are located at the interface with the large subunit[19]. Alternatively, other constraints might also lead to conserved neighborhoods. Although the elongation factor TufA physically interacts with the large ribosomal proteins L6, L11 and L14 (encoded by *rplF*, *rplK* and *rplN*, respectively[20]), *tufA* has not yet been found in the neighborhood of these genes (Fig. 1), suggesting other reasons for the context conservation. As discussed here, there is the additional level of neighborhood conservation of a conservatively rearranged uber-operon.

The existence of these conservatively rearranged gene clusters across such a wide range of species leads to the broader question of an evolutionary explanation for the pattern observed. A possible scenario is a type of ‘purifying selection’. Rearrangements of genomes occur randomly and frequently during evolution. Prokaryotic organisms maintain genes of similar function and that are involved in the same cellular process in clusters of the same transcriptional orientation and regulation (i.e. operons). The separation of these clusters would be selectively disadvantagous and would thus be eliminated from the population. Conversely, those rearrangements that placed genes within new clusters of functionally and regulatory related genes would have a relatively small impact on the fitness of the organism and could be maintained through drift and fixation.

Thus, we see the pattern of an uber-operon in which genes that have a similar function and are involved in the same processes are invariably maintained in the various neighborhoods of a finite set of genes in spite of myriad genomic rearrangements over time. Alternative explanations[21] might include a ‘selfish operon’ hypothesis that the origin and evolution of gene clusters are driven by horizontal transfer, although none of these adequately explain the pattern discussed here.

Conclusions

Although the evolutionary forces leading to the conservation of uber-operons are not yet fully understood, the concept can help in the characterization of gene function, possible operon structures and regulatory features. It also has implications for the description and classification of cellular processes. Uber-operons can be discrete entities containing a specific set and number of functionally related genes based on the evolutionary and functional constraints discussed earlier. These uber-operons

can be seen as a natural classification of cellular processes.

However, variations and continuums can also be seen. The variations we see in uber-operons, with the addition or loss of genes from a set in either a single species or entire taxa, could indicate variations between species on a cellular level. Novel additions of genes into an uber-operon in a particular species or taxon might indicate new biochemical pathways or regulatory changes in that species. Also, uber-operons might be good indicators of relationships between distinct processes within a cell. Some uber-operons probably share genes, the genes being in one uber-operon in one group of species and another uber-operon in a second. This organization could hint at the relationships and connections between processes. Thus, the uber-operons might form the basis for a natural classification of cellular functions and processes, as well as for the characterization of novel biochemical pathways in a particular species.

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Mitochondria and chloroplasts: localized and delocalized bioenergetic transduction

It is generally stated, even in textbooks, that energy transduction in oxidative or photophosphorylation can be described as proceeding through a delocalized electrochemical intermediate of protons. This is the equilibrium approach as discussed by Mitchell and Williams in 1961 (see Ref. 1) and later included by Mitchell under the term 'chemiosmosis' (see Ref. 1). The theory requires that there is a closed vesicular space with a defined proton potential between the generalized inside and the outside. Each mitochondrion and chloroplast should work then as a single unit. The theory was apparently supported by the structures of the organelles as very small bodies. The alternative, proposed simultaneously[1], is that the flow of protons kinetically controlled in local regions and limited by diffusion, although not necessarily in an enclosed space, could represent the intermediate of energy transduction in both organelles. Experimental evidence reviewed in three recent publications[2–4] now shows that the diffusion-limited, kinetically controlled description is correct. The organelles, which form mosaics, are shown to function via local domains[5]. The crucial new information is the revised structures of the organelles[4]. Mitochondria are now known to be very similar to chloroplasts. Both are very long, weaving bodies with complicated networks of cross-connected inner tubular (cristae and thylakoid) structures of even greater length than the organelle itself. There is very little chance that such organelles could act at equilibrium in a chemiosmotic sense. The very close positioning of the generating units for the proton gradient and the ATP-synthetase along the tubes makes it clear that, given the diffusion constant for protons, whether diffusion is on tube surfaces or in the solution of the tubes, local connections within domains are inevitable. Moreover, localized activity within the huge organelles has advantages in that (i) the response to energization can generate ATP locally quickly where it is needed in the cell without the lag time necessary to build the gradient in the whole organelles and the production of ATP where it is not required; and (ii) regions of the mitochondria can be used to connect locally to other (vesicular) membrane systems such as the endoplasmic reticulum[2–4] or the outer membrane at nerve cell synapses. The theory required to treat diffusion-controlled currents and potentials is well known and is described in textbooks of physiology under 'action currents in nerve fibres'. Of course, it could happen that an organelle will be activated equally everywhere as chemiosmosis proposes, but the likelihood of this is very small. Most inputs to a cell are not isotropic, and thus, it is desirable for the organelle adjacent to an input point to respond locally (i.e. nonisotropically). The description of the transduction of energy in cells needs urgent revision, so that the value of diffusion limitation over equilibration can be fully appreciated.

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