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Ancient Origin of the Tryptophan Operon and the Dynamics of Evolutionary Change†

Gary Xie,[1,2] Nemat O. Keyhani,[1]* Carol A. Bonner,[1] and Roy A. Jensen[1,2,3]*

Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611[1]; BioScience Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87544[2]; and Department of Chemistry, City College of New York, New York, New York 10031[3]

* Corresponding author. Mailing address: Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611. Fax: (352) 392-5922. Phone for Nemat Keyhani: (352) 392-2488. E-mail: nkeyhani@ufl.edu. Phone for Roy Jensen: (352) 475-3019. E-mail: rjensen@ufl.edu.

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INTRODUCTION

It has become quite apparent from the recent flood of genomic data that dynamic gene reorganization is an ongoing process (albeit of unknown significance) that distinguishes even closely related genomes. Genes that stay together within operons must resist the gene-scrambling process. Operons that embrace a complete complement of pathway-specific structural genes (whole-pathway operons), such as the ones encoding all the enzymes of tryptophan (Trp) biosynthesis or histidine biosynthesis, have a classical status in both biochemistry and molecular genetics that extends far beyond understanding these pathways per se. Such whole-pathway operons are broadly distributed among prokaryotes. However, the pathway genes may be completely scattered in some organisms, and in yet other organisms, the pathway genes may be organized into two or more "split-pathway" operons. This raises intriguing questions about what the evolutionary relationship is between whole-pathway operons, split-pathway operons, and those cases where all pathway genes are unlinked. Is it possible to deduce whether a given whole-pathway operon was an ancient innovation and therefore that operon splitting and/or gene dispersal followed in some lineages? Or are whole-pathway operons relatively recent innovations that are derived from split-pathway operons? Or, since these two scenarios are not mutually exclusive, is it possible that both apply?

An ideal operon system for this analysis is the *trp* operon. We show that the *trp* operon must have been present in early prokaryote ancestors. In *Bacteria* but not in *Archaea*, sufficient genome representation exists to deduce an ancestral whole-pathway *trp* operon. The regulation of this operon may initially have been quite minimal since the first evolutionary step(s) probably would be to collect the structural genes together. Parsimony principles support a hypothesis developed in this paper of two major evolutionary events in *Bacteria*, one splitting the ancestral operon in two and the other rejoining it by gene fusion. We assert that a detailed analysis can recognize occasional events of lateral gene transfer (LGT) or paralogy. Both are likely to be associated with Trp pathway genes engaged in specialized metabolic pathways other than primary amino acid biosynthesis. We show that when two sister lineages differ in particular *trp* operon characteristics, it is possible to deduce which is the derived change and which reflects the state of the ancestral node.

Recently, Gogarten et al. (28) endorsed a "synthesis" that will acknowledge both the traditional tree-like behavior (vertical descent of genes) and web-like, reticulate behavior (horizontal gene transfer) of the evolutionary process. They leave it open whether or not "vertical descent remains the best descriptor of the history of most genes over evolutionary time." Our overall analysis yields a very optimistic viewpoint that the evolution of the *trp* operon can be deduced as a vertical genealogy, with events of LGT and paralogy enriching the analysis as interesting features rather than undermining or obliterating the vertical trace of evolutionary history.

Biochemical Pathway of Tryptophan Biosynthesis

Nomenclature. The inconsistencies of nomenclature for designations of genes involved in aromatic biosynthesis have created increasingly awkward problems for comparative analyses, and in order to cope with genomic comparisons, we have implemented a logical system of naming *aro* genes at a level corresponding to catalytic domains (13, 31, 44, 90, 91, 88). We

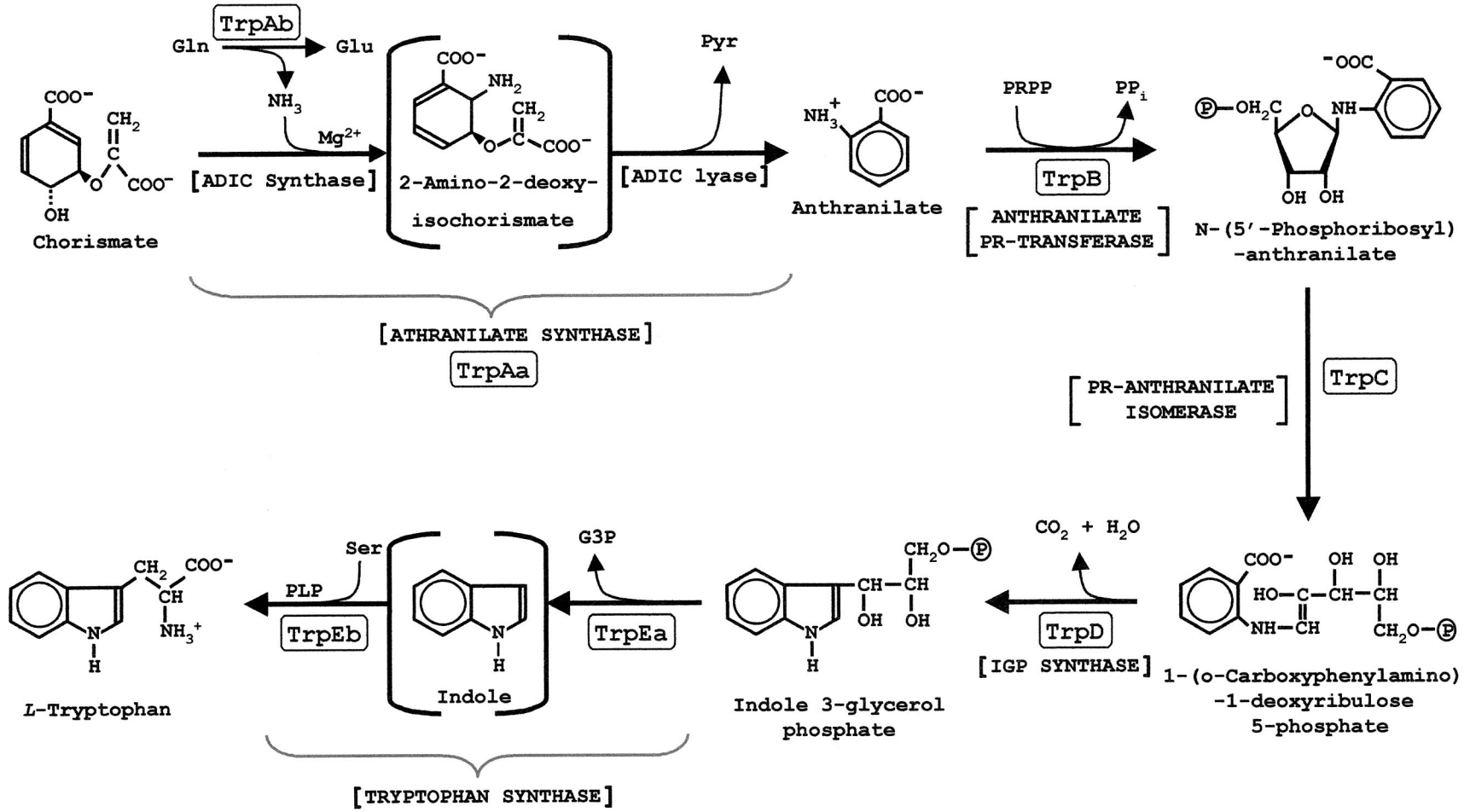


FIG. 1. Biochemical pathway of tryptophan biosynthesis. The nomenclature used in this paper for the seven catalytic domains is in boxes. See Table 1 for the alternative designations used in the literature. Anthranilate synthase catalyzes the overall reaction from chorismate to anthranilate via the half-reactions shown, whereby 2-amino-2-deoxyisochorismate (ADIC) is an enzyme-bound intermediate (62). The TrpAa/TrpAb complex functions as an amidotransferase, utilizing glutamine as the source of the *o*-amino group of anthranilate. TrpAa can catalyze the overall reaction alone in the presence of NH_3 (thereby functioning as an aminase). TrpAb alone in some cases may be able to function as a glutaminase. As shown by McDonald et al. (59), *Pseudomonas* and *Streptomyces* species form ADIC as the product of a reaction catalyzed by PhzE. PhzE has fused domains that are homologues of TrpAa and TrpAb, which we have denoted TrpAa•TrpAb_phz (93) (see Table 1). In these organisms, ADIC can be considered a branch point that proceeds to Trp on the one hand and to phenazine pigments on the other hand. Tryptophan synthase catalyzes a second overall reaction, converting indoleglycerol phosphate to Trp in a reaction path where indole is always an intermediate. The alpha (TrpEa) and beta (TrpEb) subunits catalyze the reactions shown in which the indole intermediate is processed through a tunnel (85). PR, phosphoribosyl; IGP, indoleglycerol phosphate; G3P, glyceraldehyde 3-phosphate.

TABLE 1. Key to nomenclature conversions

Function	Gene name[a]		Protein domain encoded
	Suggested	Conventional	
Tryptophan biosynthesis	*t*rpAa	*trpE*	Anthranilate synthase, aminase subunit (α)
	trpAb	*trpG*	Anthranilate synthase, amidotransferase subunit (β)
	trpB	*trpD*	Anthranilate phosphoribosyl transferase
	trpC	*trpF*	Phosphoribosyl-anthranilate isomerase
	trpD	*trpC*	Indoleglycerol phosphate synthase
	trpEa	*trpA*	Tryptophan synthase, α subunit
	trpEb	*trpB*	Tryptophan synthase, β subunit
	trpAa●*trpAb*[b]	*trpE(G)*	Fusion of first two domains above
Phenazine biosynthesis	*trpAa*●*trpAb_phz*[b]	*phzE*	ADIC synthase
Folate biosynthesis	*pabAa*	*pabB*	4-Amino-4-deoxychorismate synthase, aminase subunit (α)
	pabAb	*pabA*	4-Aminobenzoate synthase, amidotransferase subunit (β)
	pabAc	*pabC*	4-Amino-4-deoxychorismate lyase (γ) subunit

[a] Nomenclature is at the level of catalytic domain in order of reaction steps in the pathway. Overall reactions of tight complexes are assigned one capital letter, and then α, β, and γ subunits are assigned lowercase letters. *trpAa* and *pabAa* are homologs, as are *trpAb* and *pabAb*. The convention of a bullet denotes a fusion. TrpAa●TrpAb catalyzes the overall reaction of anthranilate synthase (see Fig. 1). TrpAa●TrpAb_phz is a distinct and shortened subgroup of TrpAa●TrpAb that catalyzes only the first half-reaction, ADIC synthase.

have extended this nomenclature to the Trp pathway (89, 92) (see Fig. 1 and Table 1). The two overall enzyme reactions that utilize a complex of nonidentical subunits have been denoted with lowercase letters (TrpAa and TrpEa are α-subunits for anthranilate synthase and tryptophan synthase, respectively; TrpAb and TrpEb are β subunits for anthranilate synthase and tryptophan synthase, respectively). Capital letters are assigned according to the order of the enzyme reactions (or overall reactions, in the case of the two complexes). C. Yanofsky has expressed to us his preference (probably shared by most experimentalists working specifically with *trp* systems) for adherence to previous nomenclature schemes to minimize disruption of what is most familiar in the existing literature. Admittedly, the designations generally in use for the Trp branch do not generate as many problems of annotation errors as is the case for the rest of the aromatic pathway, but for consistency with our overall work with the aromatic pathway, we use the new naming system in this paper. Both sets of designations are shown in Table 1.

Seven catalytic domains and two α/β-subunit complexes. Trp is an essential amino acid among the assemblage of required amino acids in mammals. Trp is generally synthesized by free-living prokaryotes, lower eukaryotes, and higher plants. The Trp pathway is one of three amino acid branches diverging from a common flow route that produces chorismate. The apparent universal biosynthetic pathway for Trp biosynthesis that initiates with chorismate and L-glutamine is shown in Fig. 1. Seven catalytic domains are deployed to carry out the reactions shown. In a given organism these may be individually expressed, but a wide variety of gene fusions that encode single proteins carrying two or more catalytic domains are known.

TrpAa can function as an ammonia-utilizing aminase in the anthranilate synthase reaction. Although the aminase reaction can proceed with ammonia at unphysiologically high pH values, such reactions typically rely upon a glutamine-utilizing glutaminase subunit to deliver the ammonia at the active site (probably within a "tunnel"). Accordingly, TrpAb is a glutaminase homologue that forms a complex with TrpAa, thereby conferring an amidotransferase component to the overall anthranilate synthase reaction in the presence of glutamine. In either case, whether or not the overall anthranilate synthase reaction is carried out in the presence of TrpAb, 2-amino-2-deoxyisochorismate (ADIC) is an enzyme-bound intermediate. Interestingly, some species of *Pseudomonas* and *Streptomyces* produce an enzyme called PhzE (59), which carries out the ADIC synthase reaction but not the ADIC lyase reaction (see Fig. 1). ADIC is then converted ultimately to phenazine pigments. PhzE is a fusion of domains homologous to TrpAa and TrpAb (hence our designation TrpAa●TrpAb_phz in Table 1). TrpAa belongs to a protein superfamily that includes other chorismate-utilizing enzymes: PabAa converts chorismate to 4-amino-4-deoxychorismate (precursor of 4-aminobenzoate), and MenF and EntC are different homologue subgroups that convert chorismate to isochorismate (as precursors of ubiquinones and an iron siderophore, respectively).

Tryptophan synthase also exists as a complex of nonidentical subunits and is one of the best-understood examples of allosteric interaction exerted between subunits (97). Why indole should be sequestered to a tunnel in the α/β complex of tryptophan synthase is not known, but indole is volatile and rather toxic. Yanofsky has speculated that recent findings of a role for indole in quorum sensing and biofilm formation might suggest that indole either produced by tryptophanase or otherwise available in the environment may serve as a metabolite cue that might otherwise be disrupted if biosynthetic indole were not enzyme-bound (see reference 96 and references therein). It has been speculated (92) that some *Archaea* may not form a tryptophan synthase complex.

Relatives of Trp pathway catalytic domains. The pathway of Trp biosynthesis is the first amino acid pathway for which the atomic structure of every catalytic domain has been determined (58), a circumstance of significance because evolutionary analysis can be greatly enhanced through insight gained at the structural level of protein folding. Consultation of the reference by Yanofsky et al. (97) is highly recommended for a definitive presentation of the detailed literature up to about

2000. Each catalytic domain belongs to a protein superfamily at the structural level of protein folding. Many of the catalytic domains exhibit clear homology on the criterion of amino acid identity with proteins that have different substrate specificities and which participate in different pathways. From an evolutionary perspective, this is of interest with respect to such questions as the extent to which the Trp pathway enzymes have been assembled (via gene duplication and substrate alteration) by recruitment of homologues from other pathways or the extent to which the Trp pathway has been the source of genes recruited for function in other pathways or a homologous gene with a recent history of function in another pathway has "crossed over" to replace a Trp pathway gene (or vice versa). This aspect is not addressed further in this article except indirectly (e.g., see the later section on the search for an elusive *trpC* gene).

Identical Trp pathways exist within varied metabolic contexts. The Trp pathway is generally defined as an unbranched pathway that begins with chorismate and produces Trp as a substrate for general protein synthesis. The Trp pathway appears to have evolved only once. These aspects of universality are favorable for the task of deducing the evolutionary history. However, many aspects of biochemical individuality are not usually considered. In some cases, Trp biosynthesis does not compete with Phe and/or Tyr biosynthesis because one or both of these are absent. In other cases, as exemplified by the use of ADIC for phenazine biosynthesis in *Pseudomonas* and *Streptomyces* species, chorismate is no longer the last branch point, and if one starts with chorismate as a reference point, then the pathway is branched. The pathway does not necessarily end exclusively with the Trp end product supplying protein synthesis, e.g., in cases where Trp may be a component of an antibiotic (as in *Streptomyces*), or where it is converted to indoleacetic acid in plant symbionts such as *Azospirillum*. Eukaryotes (but no prokaryotes so far) deploy Trp as a precursor of niacin. In such cases, the pathway can be considered divergently branched at the end, with Trp being guided to different molecular fates.

Trp is the most biochemically expensive of the amino acid pathways, requiring the input of erythrose-4-phosphate, ATP, phosphoribosyl pyrophosphate (PRPP), two phosphoenolpyruvate molecules, L-glutamine, and L-serine. Thus, efficient regulation is generally expected, but these rules no longer apply in an endosymbiont such as *Buchnera*, which has abandoned Trp regulation. In this case, loss of regulation can be viewed as a positive selective step in order to satisfy the needs of its aphid host. In addition, some prokaryotes sustain different physiological or developmental states where the demands impacting the Trp pathway may be more complicated than just sensing the availability of Trp for protein synthesis. These often involve specialized pathways that coexist with primary Trp biosynthesis. These specialized pathways are encoded in part or entirely by divergent *trp* gene duplicates whose expression is triggered by a variety of temporal and environmental cues, e.g., to make a given pigment or antibiotic derived in part from the Trp pathway.

These are all interesting but complicating elements that we have tried to keep in mind. This is relevant to the task of sorting out and recognizing paralogues (or xenologues) that may be engaged in specialist pathways other than primary Trp biosynthesis. Appreciation of such complexity may also prove relevant to understanding the nature of split-pathway *trp* operons in many prokaryotes.

Operon Stability

Most molecular biologists who are familiar with the elaborate control features of well-studied operon systems would feel a strong conviction that once evolved, these would resist change (at least disruptive changes). Yet a bioinformatic analysis of the then-available genomes in 1999 (37) produced the conclusion that operon structures, such as the *trp* operon, are unstable, as inferred from observations of extraneous insertions, gene dispersal, and scrambling of gene order within operons. Characterization of operons as unstable connotes susceptibility to deterioration. If true, this implies that the selective advantages conferred by such operons must be weak.

The Itoh et al. study (37) was a broad-scope analysis of many operons that was necessarily limited with respect to in-depth consideration of any individual operon system. It should be noted that for these kinds of studies, operons have been considered simply as a collection of structural genes that are linked. The presence or absence of linked or unlinked regulatory elements has not usually been evaluated, undoubtedly because this is not easily done. In this paper we pursue in great detail the evolution of a single well-known operon system in the large number of prokaryote genomes now available. We found strong support for the hypothesis that the *trp* operon, minimally defined as the linked assemblage of structural genes for tryptophan (Trp) biosynthesis, is of ancient origin and has indeed followed a dynamic time course of change that includes several identifiable milestone events in *Bacteria*. Our study leads to the further hypothesis that the instability of early *trp* operons (and perhaps some modern ones) can be attributed to weak positive selection conferred by relatively undeveloped control mechanisms.

We suggest that since the time that operons evolved a variety of control mechanisms, the characterization of operons as dynamic (rather than unstable) yields better semantics to describe a positive ongoing process of fine-tuning. In modern free-living organisms, the variety of recently evolved *trp* operon systems which differ from one another and are endowed with intricate control features mediated by one or more unlinked regulatory genes may in fact be highly stable in the contemporary time frame. One caveat, however, is that this frequently will not apply to pathogenic or endosymbiotic relatives, where the rules dictating selective advantage have completely changed.

trp Operon and Its Regulation

The biochemical pathway of Trp biosynthesis is a classical system of biochemical genetics (95, 97). In *Escherichia coli* the component genes are organized into a single transcriptional unit to form the *trp* operon. (This is not strictly correct to the extent that a weak internal promoter exists.) The Trp pathway has become one of the most intensely studied systems in biology, thanks largely to the truly Herculean labors of C. Yanofsky and his many students and colleagues. This system has produced knowledge that extends well beyond the details of the Trp pathway per se, e.g., proof of codon and amino acid

colinearity and an early precedent for attenuation mechanisms (95, 96). The individual reactions of Trp biosynthesis are invariant, but experimental work with a variety of organisms reveals substantial diversity with respect to gene fusion, gene organization, and mechanisms of regulation.

Known regulatory mechanisms. At the bioinformatic level, the analysis of *trp* operons in the literature has been largely restricted to the structural genes. Consideration of regulatory features has been understandably limited, mainly because relatively little comparative information is available at the experimental level and also because analysis of alternative stem-loop structures, etc., is not a trivial task. *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Lactococcus lactis* represent clades for which detailed control mechanisms have been described, each of them entirely different. Importantly, each mechanism seems to be narrowly distributed, and therefore we infer that they are of recent origin. Note that in each case, unlinked genes exist that markedly decrease the probability that the total regulated operon system could be transferred by LGT in one event.

Regulation of Trp biosynthesis in *E. coli*, the most widely known system, is quite sophisticated (23, 94), being subject to the following multiple levels of control: (i) repression control via the Trp repressor (encoded by the unlinked *trpR*) which binds Trp as a corepressor moiety, (ii) an attenuation mechanism mediated by a Trp-rich leader peptide (encoded by *trpL*), and (iii) allosteric feedback inhibition of anthranilate synthase by Trp (95). The *E. coli* mechanisms of overall *trp* operon regulation are generally shared by the enteric lineage of *Bacteria*, defined by us as the clade that includes *Shewanella putrefaciens* as the outlying point of divergence from *E. coli*.

Bacillus subtilis has a different system of *trp* operon regulation (72, 80, 95, 96), whereby genes unlinked to the *trp* operon encode (i) a *trp* RNA-binding attenuation protein (TRAP) encoded by *mtrB* as well as (ii) an anti-TRAP gene product encoded by *rtpA* (80). Trp both feedback inhibits anthranilate synthase and activates TRAP for attenuator function, whereas uncharged $tRNA^{Trp}$ induces synthesis of anti-TRAP. TRAP can also block translation of the *trp* operon through interference with the ribosome-binding site. The clade sharing the TRAP system of regulation includes *Bacillus halodurans*, *Bacillus stearothermophilus*, and *Oceanobacillus iheyensis* in addition to *Bacillus subtilis*. At this time it is not clear whether the anti-TRAP component is present throughout this clade.

A third finely tuned system of regulation has been documented in *Lactococcus lactis* (69). In this case uncharged tRNA can bind directly to the leader transcript, stabilizing an antiterminator configuration that promotes expression of the operonic genes. In *Lactococcus lactis*, unlinked, unknown genes involved in *trp* operon transcript processing and in transcription initiation have been suggested (69). The presence or absence of the *Lactococcus lactis* mode of *trp* operon regulation in close relatives, such as species of *Streptococcus*, has apparently not yet been investigated.

In *Pseudomonas aeruginosa*, the fourth well-documented system, the Trp pathway is represented by four operon entities: a free-standing *trpAa*, the *trpAbBD* operon, a free-standing *trpC*, and the *trpEbTrpEa* operon. The *trpAa* and *trpAbBD* operons are regulated by attenuation mechanisms employing leader peptides (67), whereas the *trpEbtrpEa* operon is controlled by an indoleglycerol phosphate-activated regulatory protein encoded by *trpI* (6). *trpC* is not known to be regulated in any way. The *P. aeruginosa* system is complicated by the presence of paralogues of *trpAa* and *trpAb*. These include genes of unknown physiological function (also known as *phnA* and *phnB*) expressed in stationary phase (57) as well as two copies of PhzE (*trpAa•trpAb_phz*), a gene that encodes ADIC synthase (Fig. 1), the initial reaction committed to phenazine biosynthesis. It is not entirely clear what physiological conditions exist in *P. aeruginosa* (and close relatives) that have resulted in its unusual use of indoleglycerol phosphate as a regulatory cue for the selective regulation of the *trpEbTrpEa* operon, but it is certainly evident that much has been committed to the overall regulation in this system. Close genomic neighbors of *P. aeruginosa* that possess identical split-pathway *trp* operons and *trpI* include *Pseudomonas fluorescens*, *Pseudomonas syringae*, and *Azotobacter vinelandii.*

Unknown regulatory systems awaiting discovery? We do not know the extent to which the total network of regulatory elements governing the single *trp* operons in the *E. coli*, *B. subtilis*, and *L. lactis* clades or the multiple split-pathway operons of the *P. aeruginosa* clade might be more elaborate than that of most other organisms. Different lifestyles undoubtedly select mechanisms accommodating varied ranges of control responsiveness. A simple mode of Trp regulation may very well be appropriate in a cyanobacterium but not *E. coli*. A variety of alternative regulatory systems in other modern lineages probably remain to be elucidated. Transcriptional regulation has been reported in the whole-pathway operons of *Methanobacterium thermoautotrophicum* (26) and *Pyrococcus kodakaraensis* (77), but the exact mechanisms are unknown. The split-pathway operons of the clade represented by *Rhizobium meliloti* (7) and *Azospirillum brasilense* (21) exhibit an attenuation mechanism involving a Trp-rich leader peptide, upstream of the *trpAa•trpAb* fusion, but no regulation of the remaining two partial-pathway operons is known. Physically separated split-pathway *trp* operons may be of positive selective value per se for presently unknown reasons, whereby it might be of value to discoordinate the expression of some *trp* genes from others, or they may simply be the outcome of initially disrupted whole-pathway operons that subsequently recruited a refined control mechanism accommodating the gene separations.

Feasibility for Deduction of Evolutionary Histories

The current database for prokaryotes, at least for the *Bacteria*, now has sufficient genome representation to accommodate systematic attempts to deduce the evolutionary history of well-understood biochemical pathways. Such an effort requires the successful recognition and confrontation of complications such as (i) irregular genome expansions in the form of the unpredictable emergence of new paralogues or analogues, (ii) an erratic and differential phylogenetic loss of paralogue genes (often the cause of what has been termed unrecognized paralogy), and loss of analogue genes (could be termed unrecognized analogy), and (iii) lateral (horizontal) gene transfer

TABLE 2. Cross-reference guide to organisms and figures

Prokaryote	Figure(s) in which it appears
Aeropyrum pernix	2A, 4, 5, 10
Anabaena sp.	2C, 4, 6A
Aquifex aeolicus	2A, 4
Archaeoglobus fulgidus	2A, 4, 5, 10
Azospirillum brasilense	4, 9
Azotobacter vinelandii	9
Bacillus anthracis	2B, 4, 11
Bacillus halodurans	2B, 11
Bacillus stearothermophilus	2B, 4, 11
Bacillus subtilis	2B, 4, 6A, 11
Bordetella pertussis	2D, 4, 6B, 9
Borrelia burgdorferi	2C
Bradyrhizobium japonicum	9
Brucella melitensis	2D, 4, 9
Buchnera sp.	8
Buchnera aphidicola	2D, 4
Burkholderia fungorum	2D, 6B, 9
Burkholderia pseudomallei	4
Campylobacter jejuni	2D, 4, 6A
Caulobacter crescentus	2D, 4, 6B, 9
Chlamydia muridarum	2C
Chlamydia trachomatis	2C
Chlamydophila pneumoniae	2C
Chlamydophila psittaci	2C, 4, 6A
Chlorobium tepidum	2C, 4
Chloroflexus aurantiacus	4
Clostridium acetobutylicum	2B, 4, 6A
Clostridium difficile	2B
Corynebacterium diphtheriae	2B, 3, 4
Corynebacterium glutamicum	3, 4
Coxiella burnetii	2D, 6B, 9
Cytophaga hutchinsonii	2C, 6A
Dehalococcoides ethenogenes	2A, 6A
Deinococcus radiodurans	2A, 4, 6A
Desulfovibrio vulgaris	2D, 4, 6A
Desulfitobacterium hafniense	6A
Enterococcus faecalis	2B, 7
Escherichia coli	2D, 4, 6B, 8
Ferroplasma acidarmanus	2A, 5, 10
Geobacter sulfurreducens	2D, 4, 6A
Haemophilus actinomycetemcomitans	4, 8
Haemophilus ducreyi	2D, 8
Haemophilus influenzae	2D, 4, 8
Halobacterium sp.	2A, 4, 5, 10
Helicobacter pylori	2D, 4, 6A
Klebsiella pneumoniae	4, 8
Lactococcus lactis	2B, 4, 7
Legionella pneumophila	2D, 4, 6B, 9
Listeria monocytogenes	2B, 7, 11
Magnetospirillum magnetotacticum	2D, 6B, 9
Magnetococcus sp.	6B, 9
Mesorhizobium loti	4
Methanobacterium thermoautotrophicum	2A, 4, 5, 10
Methanococcus jannaschii	2A, 4, 5, 10
Methanosarcina barkeri	2A, 5, 10
Methylococcus capsulatus	2D, 6B
Mycobacterium avium	2B, 3, 4
Mycobacterium bovis	2B, 3
Mycobacterium leprae	2B, 3
Mycobacterium smegmatis	2B, 3
Mycobacterium tuberculosis	2B, 3, 4, 6A
Mycoplasma genitalium	2B, 7
Mycoplasma pneumoniae	2B, 7
Neisseria gonorrhoeae	4, 9
Neisseria meningitidis	2D, 4, 6B, 9
Nitrosomonas europaea	2D, 6B, 9
Nostoc punctiforme	2C, 4
Oceanobacillus iheyensis	11
Pasteurella multocida	2D, 4, 8
Porphyromonas gingivalis	2C
Pseudomonas aeruginosa	2D, 4, 6B, 9
Pseudomonas fluorescens	9
Pseudomonas putida	4
Pseudomonas syringae	2D, 4, 9
Prochlorococcus marinus	2C
Pyrobaculum aerophilum	2A, 5, 10
Pyrococcus abyssi	2A, 4, 5, 10
Pyrococcus furiosus	2A, 4, 5, 10
Pyrococcus horikoshii	2A, 5, 10
Ralstonia metallidurans	2D, 6B, 9
Rhizobium loti	2D, 9
Rhizobium meliloti	4
Rhodobacter capsulatus	9
Rhodobacter sphaeroides	2D, 6B
Rhodopseudomonas palustris	2D, 4, 6B, 9
Rickettsia prowazekii	2D
Salmonella enterica	2D, 4, 8
Shewanella putrefaciens	2D, 4, 6B, 8
Sinorhizobium meliloti	2D, 9
Sphingomonas aromaticivorans	2D, 6B
Staphylococcus aureus	2B, 4, 11
Staphylococcus epidermidis	2B, 5, 11
Streptococcus equi	2B, 7
Streptococcus gordonii	2B, 7
Streptococcus mutans	2B, 4, 7
Streptococcus pneumoniae	2B, 4, 6A, 7
Streptococcus pyogenes	2B, 7
Streptomyces coelicolor	2B, 3, 4
Sulfolobus solfataricus	2A, 4, 5, 10
Synechococcus sp.	2C
Synechocystis sp.	2C, 4
Thermomonospora fusca	2B, 3, 4
Thermoplasma acidophilum	2A, 4, 5, 10
Thermoplasma volcanium	5, 10
Thermotoga maritima	2A, 4, 6A
Thiobacillus ferrooxidans	2D, 4, 6B, 9
Treponema denticola	2C
Treponema pallidum	2C
Ureaplasma urealyticum	2B, 7
Vibrio cholerae	2D, 4, 8
Wolbachia sp.	2D
Xanthomonas campestris	9
Xanthomonas axonopodis	9
Xylella fastidiosa	2D, 4, 6B, 9
Yersinia pestis	2D, 4, 8

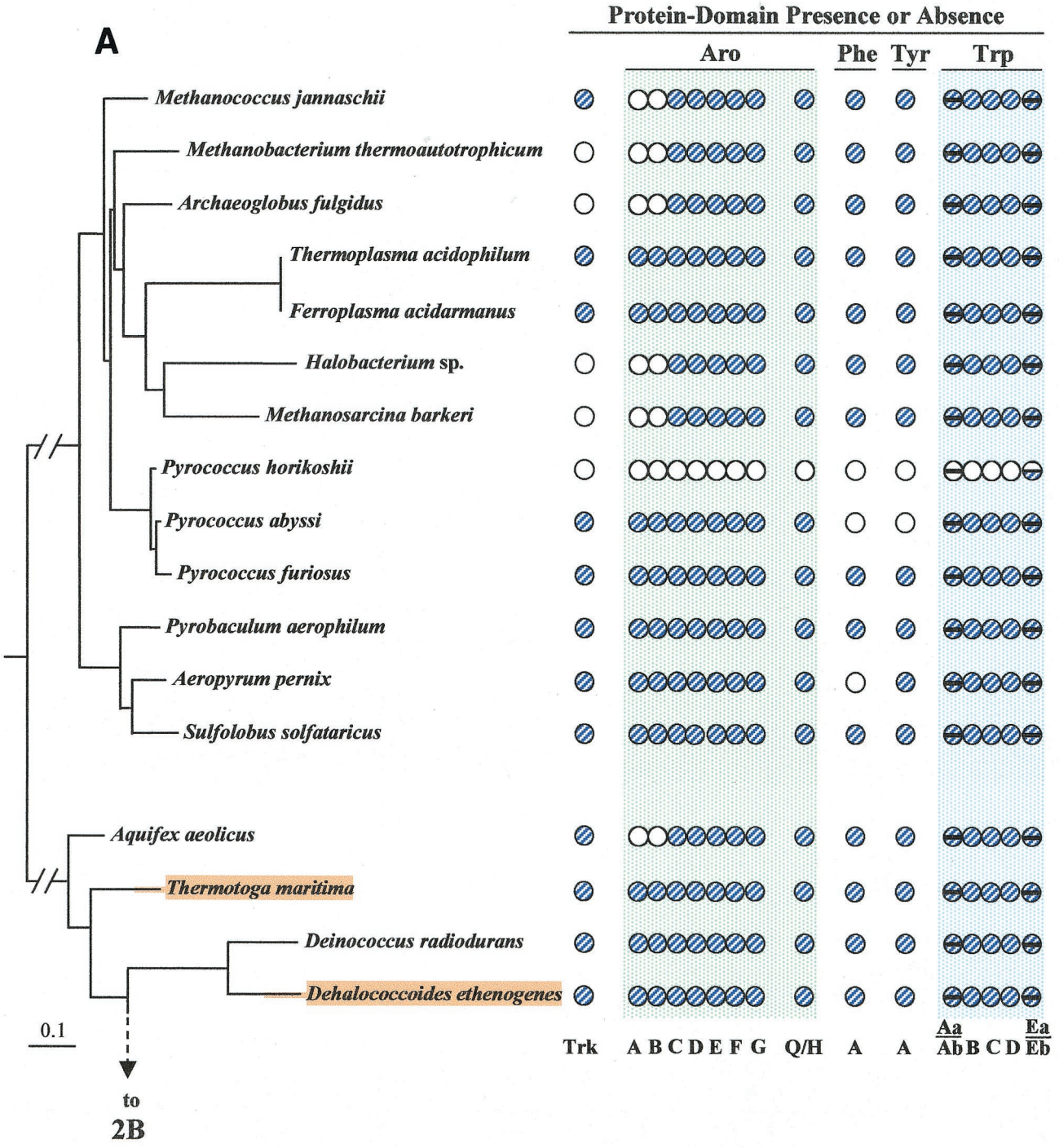
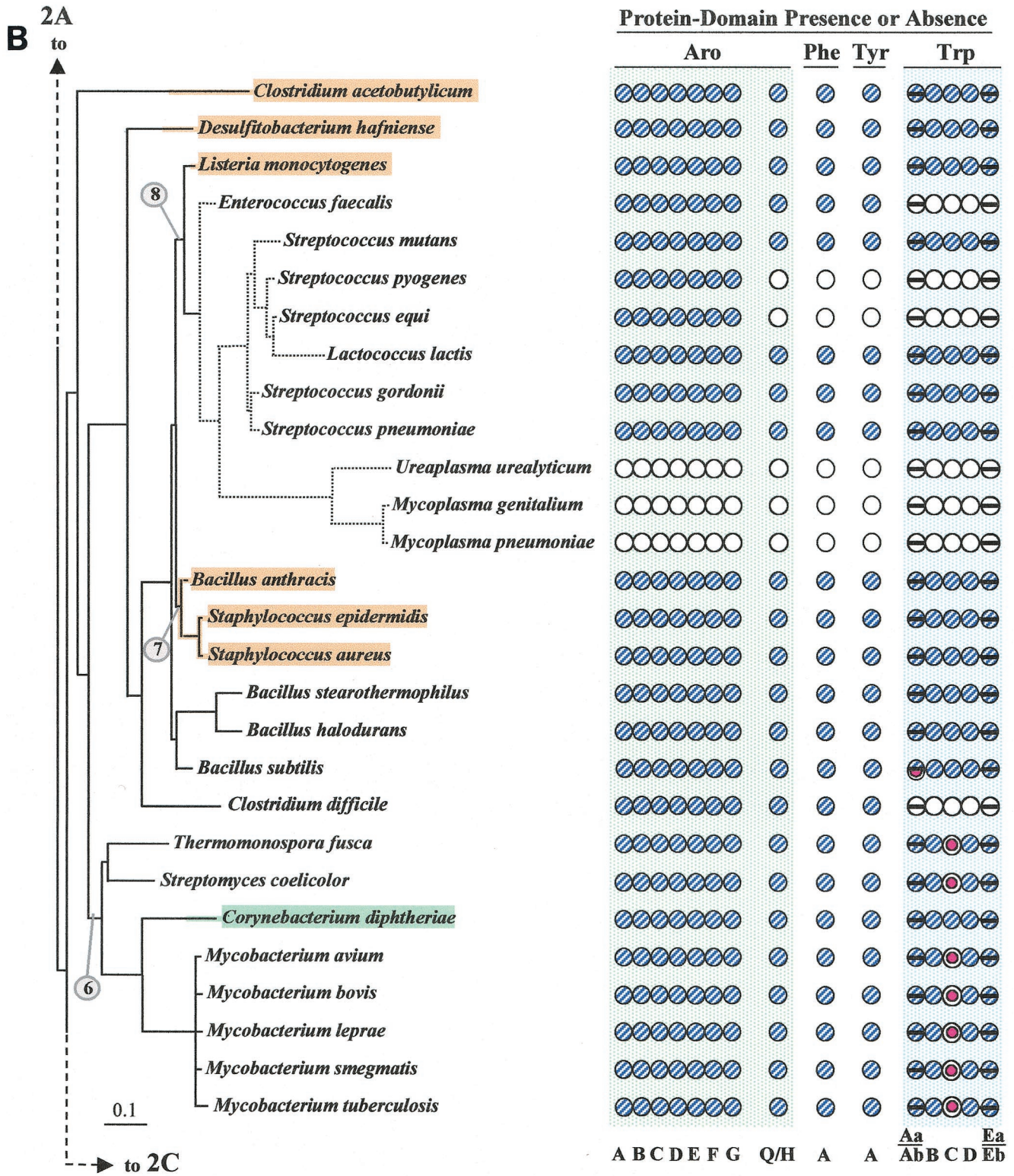


FIG. 2. Distribution of aromatic-pathway catalytic domains among prokaryotes. In each panel, 16S rRNA trees are shown at the left, and the presence (shaded circles) or absence (open circles) of domains is shown at the right. Note that only the presence or absence of genes, not gene order, is indicated. Catalytic domains of the common trunk of aromatic biosynthesis (Aro), the phenylalanine branch (Phe), the tyrosine branch (Tyr), and the Trp branch are labeled across the top right; the specific letter designation for a given domain is shown at the bottom. In the Trp grouping, split circles are used to indicate the presence or absence of TrpAa (top half-circle) and TrpAb (bottom half-circle) or TrpEa (top half-circle) and TrpEa (bottom half-circle). In panel A, the presence or absence of transketolase (Trk) is indicated by the left column of circles. The connecting point of a tree segment in any given panel (A, B, C, and D) with a tree segment(s) in another panel is marked with a broken line. The scale bar corresponds to substitutions per site. Dotted lines in the *Streptococcus* region (B) and the *Buchnera* region (D) indicate our suggestion that the 16S rRNA tree shown may not reflect exactly the correct order of branching, and perhaps these organisms branch from a slightly deeper position. See Fig. 8 for the suggested branching order of *Buchnera*. Circled numbers indicate eight node positions from which Trp protein trees are congruent with the 16S rRNA tree. The common trunk of aromatic biosynthesis is encoded by seven genes whose corresponding gene products are named AroA through AroG. The common-pathway genes are named in exact order of pathway reactions according to the precedent implemented in references 12, 31, 76, 90, and 91. The chorismate mutase block is represented by homologues of either AroQ (usually) or AroH (seldom) (12). PheA refers to prephenate dehydratase, the sequence of the relatively infrequent arogenate dehydratase being currently unknown. TyrA refers to a homologue family that includes prephenate dehydrogenase, arogenate dehydrogenase, or cyclohexadienyl dehydrogenase (9, 88). See Fig. 1 for details of Trp biosynthesis. The names of organisms



retaining the putative ancestral whole-pathway *trp* operon are shaded orange, those having the two split-pathway operons are shaded magenta, and those having operons rejoined by fusion of *trpD* and *trpC* are shaded aqua. These correspond to the major evolutionary events portrayed in Fig. 12 and indicated with the same color-coding scheme. Probable pseudogenes in chlamydiae (C) and *Coxiella* (21) are indicated with heavy black slash marks. Genes that function in two pathways (*trpAb* in *Bacillus subtilis* and *trpC* in actinomycete bacteria) are marked with magenta bull's-eyes in B. Panel A includes the *Archaea* and a few of the deeper-branching *Bacteria* at the bottom. Panel B includes the gram-positive *Bacteria*. Panel C includes cyanobacteria, chlamydiae, and other organisms on the 16S rRNA tree between the gram-positive organisms in panel B and the organisms in panel D, which contains the gram-negative subdivisions of the Proteobacteria. *Wolbachia* sp. (panel D) is an endosymbiont of *Brugia malayi*. A cross-index of all organisms shown in both this figure and the remaining figures is given in Table 2.

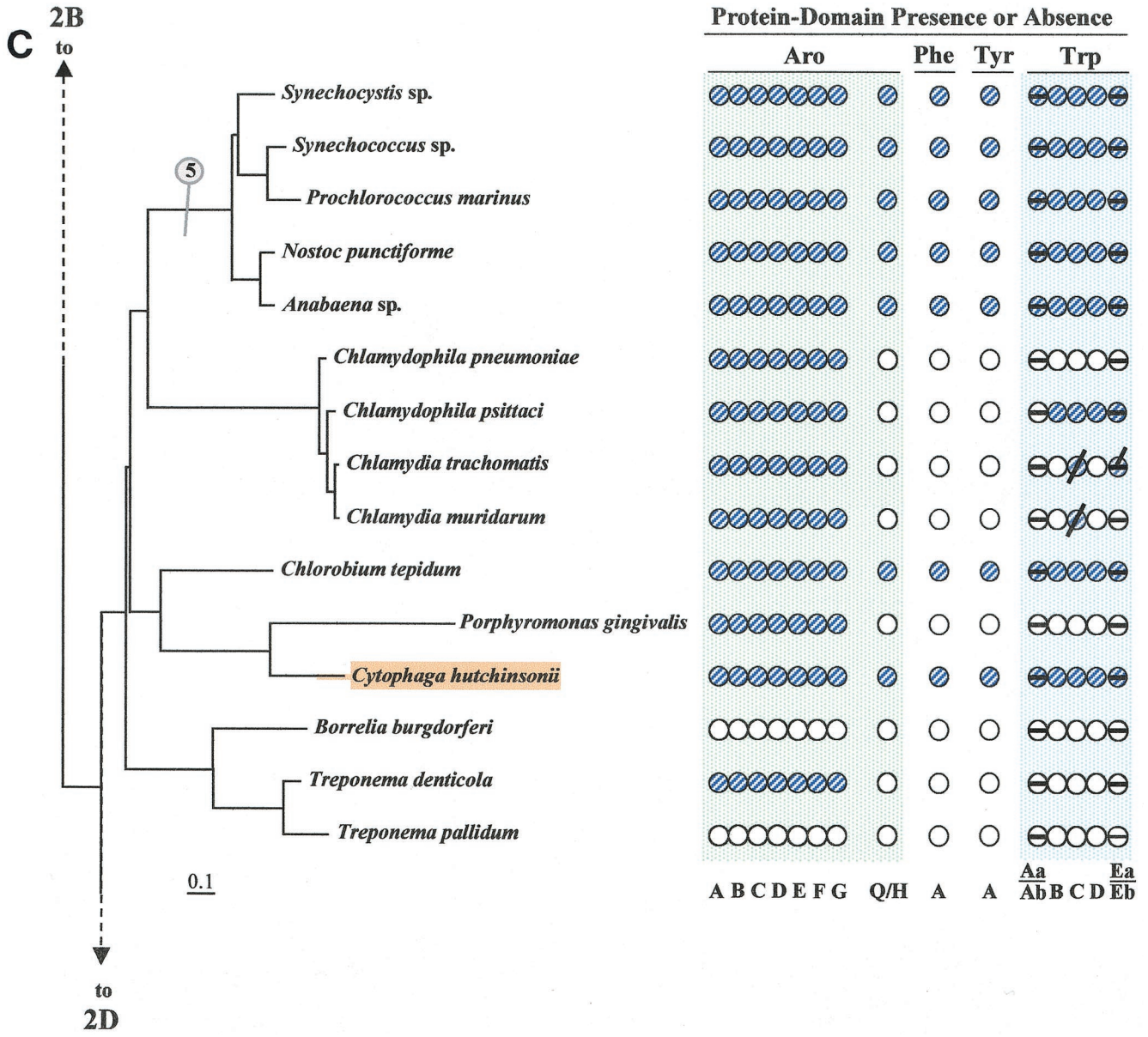


FIG. 2—*Continued.*

(LGT). Errors and inconsistencies of database annotation as well as idiosyncrasies of nomenclature can create formidable hurdles for those who are not completely familiar with a given pathway and its scholarly literature. Global computational surveys to date are simply not very informative, and the algorithms employed for automated annotation have too many limitations. For example, a very recent effort at computational identification of operons in microbial genomes (98) chose to highlight the results of *trp* operon analysis as a prime example of the analysis. However, the results presented are not comprehensive and contain serious mistakes, most likely due to errors in annotation and confusing nomenclature issues that have been perpetuated in the databases.

As a first step toward deducing the evolutionary history of overall aromatic biosynthesis, we selected the Trp branch as a challenging but manageable metabolic segment for initial analysis. Trp pathway genes have sometimes been recruited for function in specialized biochemical pathways, and ancient paralogues or xenologues may coexist with the Trp pathway genes that are engaged in primary biosynthesis. We have shown (93) that detailed case-by-case analysis can distinguish ancient *trp* paralogues (or xenologues) from their homologues engaged in primary Trp biosynthesis. A comparable study in the literature produced a detailed analysis of homologues of ornithine carbamoyltransferase in which the challenges to tracking a vertical path of evolutionary descent that are caused by the complexities of xenology and ancient paralogy were sorted out (73). This study was preceded by an analysis (49) showing that ornithine carbamoyltransferases in turn belong to a larger protein family in which the ornithine and aspartate carbamoyltransferases are very ancient paralogues. The conclusions such comprehensive studies are consistent with the

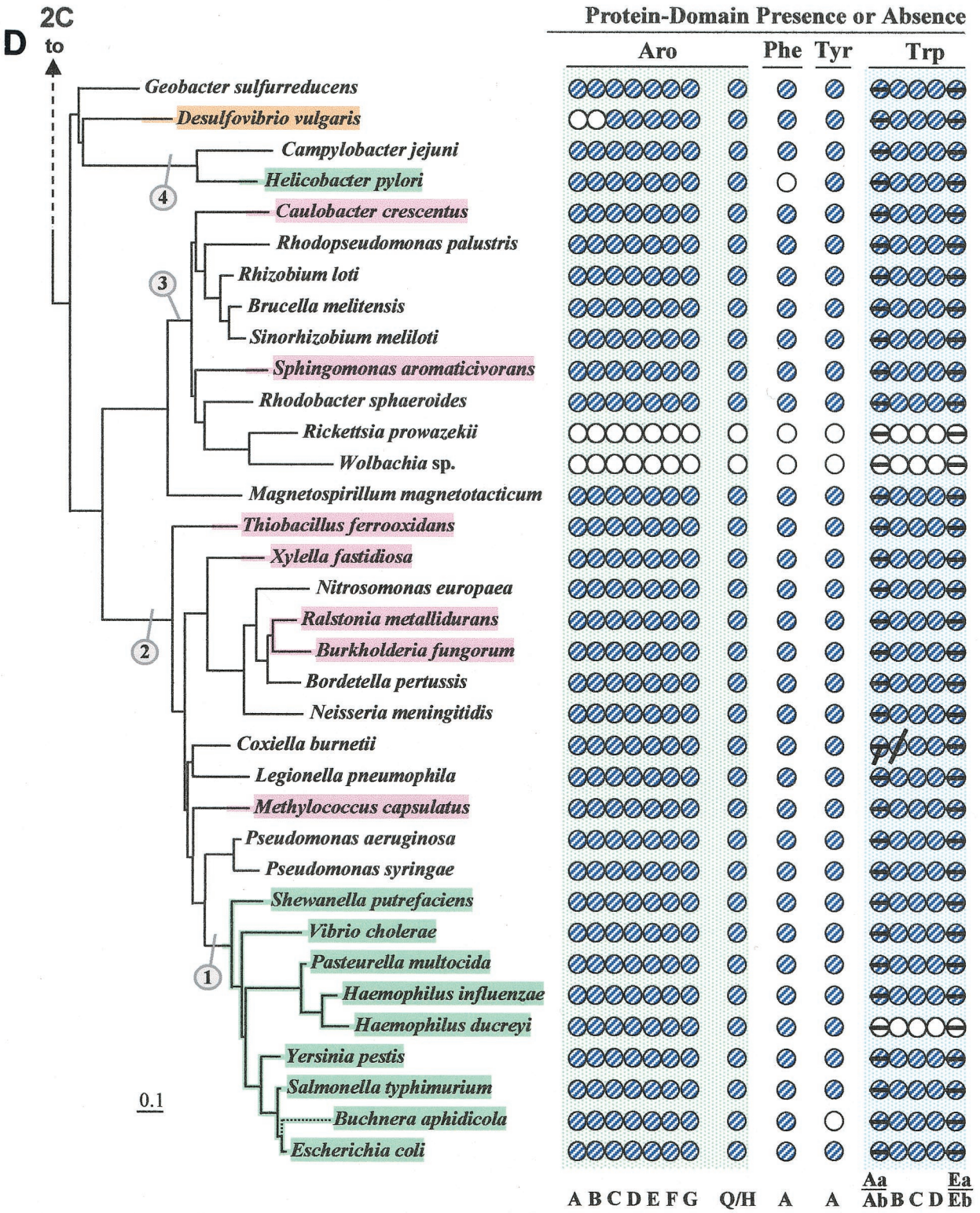


FIG. 2—*Continued.*

contentions of Glansdorff (27) and Woese (87) that complications of ancient paralogy, ancient analogy, and lateral gene transfer can be recognized sufficiently well to allow the events of vertical ancestry to be tracked.

Here we present results from an in-depth, manual analysis of Trp pathway genes in over 100 genomes. A limited amount of information is also given to illustrate the very important perspective that the evolutionary relationships of Trp biosynthesis will ultimately be best understood in its larger context as one branch of a highly divergent pathway responsible for the bio-

synthesis of aromatic amino acids as well as many other important metabolites.

GENOMIC DISTRIBUTION OF THE TRYPTOPHAN PATHWAY

Mapping of *trp* Gene Patterns to the 16S rRNA Tree

In most of the figures used in this paper, patterns of operonic gene arrangement in a given organism are mapped to the placement of that organism on a 16S rRNA tree. Table 2 keys each organism examined to the figures that show *trp* gene patterns in that organism. To what extent do the individual Trp protein trees parallel the 16S rRNA tree? It is well known that, unlike information-rich 16S rRNA, most individual proteins cannot be expected to yield robust phylogenetic trees in which the order of branching is well supported by high bootstrap values, at least not over wide phylogenetic ranges. However, in relatively narrow phylogenetic spans, we have found congruity between Trp protein trees and 16S rRNA trees (except for TrpAb, which is too small).

Figure 2 illustrates (see shaded and circled numbers) eight clades where reasonably good congruity is observed: a *Listeria*/*Bacillus*/*Staphylococcus*/*Streptococcus* grouping (Fig. 2B), *B. subtilis*, *B. stearothermophilus*, *B. halodurans* (Fig. 2B), actinomycete bacteria (Fig. 2B) cyanobacteria (Fig. 2C), *Campylobacter*/*Helicobacter* (Fig. 2D), Proteobacteria between *Caulobacter crescentus* and *Rhodobacter sphaeroides* in Fig. 2D, the clade between *Thiobacillus ferrooxidans* and *Pseudomonas syringae* (Fig. 2D), and the enteric lineage between *Shewanella putrefaciens* and *Escherichia coli* in Fig. 2D. These are all groups for which a sufficient number of closely related genomes have been sequenced. We expect that when genome sequences become available in more sparsely represented areas, e.g., around *Chlorobium* (Fig. 2C) or *Thermotoga* (Fig. 2A), additional phylogenetic spans will be congruent. Within a relatively narrow phylogenetic span, protein trees actually have the potential to discriminate branching order better than 16S rRNA trees. Our Trp protein trees (data available upon request), together with a variety of other aromatic-pathway information (see also the section on nested gene fusions), suggest that the *Enterococcus*/*Streptococcus*/*Lactococcus* grouping is outside the *Listeria*/*Bacillus*/*Staphylococcus* clade rather than within it, as shown on the 16S rRNA tree of Fig. 2B.

Assembly of these protein trees is not a trivial task because divergent paralogues or xenologues engaged in specialized metabolic activities as well some genes originating by LGT must be recognized and sorted out. Examples are given in this paper.

Trp Biosynthesis in Its Larger Context of Aromatic Biosynthesis

Trp biosynthesis is usually described as the branch of aromatic biosynthesis (reference 10 is a comprehensive biochemical review) that begins with chorismate and L-glutamine as initial substrates. In view of the fact that chorismate is not generally available from the environment as a stable nutrient, Trp biosynthesis can be considered from an in vivo perspective to initiate further upstream via the enzymatic condensation of erythrose-4-phosphate and phosphoenolpyruvate. This step is common to the biosynthesis of all three aromatic amino acids and is positioned at a point of interface with carbohydrate metabolism.

The multipurpose Fig. 2 provides a summary of the presence or absence of Trp pathway genes in the larger context of the presence or absence of genes specifying the common aromatic trunk and the sister phenylalanine and tyrosine branches. The circles in Fig. 2 from left to right represent catalytic domains (specified at the bottom of each panel) corresponding to the seven common-pathway steps (*aroA* through *aroG*), chorismate mutase (*aroQ* or *aroH*) (which is common to the short Phe and Tyr branches), and the seven catalytic domains of the Trp pathway (Fig. 1 and Table 1).

The key enzyme of Phe biosynthesis is PheA, and the key enzyme of Tyr biosynthesis is TyrA. The Phe and Tyr branches each utilize an aminotransferase step, not shown as a circle because of bioinformatic difficulties associated with deducing the substrate specificity of multiple and ubiquitous broad-specificity aminotransferases (42). Most intermediary metabolites of aromatic biosynthesis are not likely to be available from the environment; only quinate, shikimate, and anthranilate, all abundant in nature (10), are feasible precursors of Trp. Although these metabolites are indeed readily utilized when available, no prokaryotes have yet been found to rely on an exogenous source of quinate, shikimate, or anthranilate as exclusive and obligatory beginning precursors. One interesting special-case exception is *Chlamydophila psittaci*, an obligate intracellular parasite that utilizes host-derived anthranilate as a required Trp precursor (89).

Implications of Missing Genes

Unidentified analogue genes. The most obvious explanation for "missing" genes that leave a gap in an otherwise intact pathway is the existence of analogue genes, i.e., functionally equivalent genes that lack homology with the genes used to query the databases. The common pathway of aromatic biosynthesis is a good example of nonhomologous genes producing enzymes that catalyze the same reaction. These include the first step ([3-deoxy-D-arabino-heptulosonate-7-phosphate] [DAHP] synthase) (31, 44), the third step (dehydroquinase) (13), and the fifth step (shikimate kinase) (13, 18). Chorismate mutase is represented in nature by three analogue genes (13). No analogue genes are presently known for Trp pathway genes except for *trpC* (9).

Alternative metabolic relationships. In contrast to the apparent universality of the specific Trp branch, alternative enzyme steps appear to exist in nature for the Phe and Tyr branches as well as for the common trunk of aromatic biosynthesis. Some *Archaea* (Fig. 2A) and two widely spaced members of the *Bacteria* (*Aquifex* and *Desulfovibrio*, Fig. 2A and 2D) lack both AroA and AroB. Transketolase (Trk), required for generation of a substrate for AroA, is also shown in Fig. 2A because most (but not all) organisms that lack AroA and AroB also lack transketolase. (*Desulfovibrio vulgaris* [Fig. 2D] does have transketolase.) In the last six organisms, dehydroquinate, the substrate of AroC, presumably connects with carbohydrate metabolism in some unknown way that does not involve AroB or any of the known AroA homology groupings $AroA_I\alpha$, $AroA_{I\beta}$, or $AroA_{II}$ (31, 44, 76). Some support for this putative

alternative metabolic connection, based on tracer methodology, exists in the literature (79). It is also possible that quinate, either from the environment or arising endogenously in some unknown way, could be the source of dehydroquinate via the action of a quinate dehydrogenase.

Although species of *Chlamydophila* and *Chlamydia* are very close phylogenetically, the presence of Trp pathway genes varies from complete absence in *C. pneumoniae* to almost all present in *C. psittaci*. It appears that the Trp pathway in *C. trachomatis* and *C. muridarum* is in a contemporary process of reductive evolution, and the few remaining genes may be remnants (25, 89). In contrast to these species, an "incomplete" *trp* operon in *C. psittaci* appears to play a role in the capture of host kynurenine derived from tryptophan (89). Although *C. psittaci* does lack *trpAa* and *trpAb*, the remaining five *trp* genes coexist in an operon into which two novel genes have been recruited. These encode kynureninase and PRPP synthase. This creates the ability to generate PRPP (needed for the TrpB step) and to intercept host kynurenine as a source of anthranilate, cycling host-catabolized Trp back to Trp in the intracellular parasite (89). Effectively, a host-pathogen metabolic mosaic has been created, and the variant operon generates a kynurenine-to-Trp flow route instead of the usual chorismate-to-Trp flow route.

As explained above, the absence of *trpAa* and *trpAb* in *C. psittaci* is by design, and the remaining Trp pathway is functional. The likelihood that *aroA* and *aroB*, which are absent in some organisms, will prove to reflect either a new metabolic connection or the existence of unknown analogue genes has already been mentioned. In a few cases *tyrA* or *pheA* was the only aromatic-pathway gene not found by homology search. The endosymbiont *Buchnera* (Fig. 2D), which lacks *tyrA*, may not need to synthesize tyrosine because the host has phenylalanine hydroxylase, which can convert phenylalanine to tyrosine. *Aeropyrum pernix* (Fig. 2A) and *Helicobacter pylori* (Fig. 2D), which both lack *pheA*, may very well possess arogenate dehydratase, an alternative pathway step for prephenate dehydratase (reference 39 and references therein). No gene encoding an arogenate dehydratase has yet been cloned and sequenced.

Reductive evolution. Reductive evolution is descriptive of the process in which pathogens or symbionts decrease genome size by abandoning genes that are needed by their free-living relatives but dispensable because of the availability of resources from a host or symbiont partner. The genus *Pyrococcus* exhibits marked variation in the capability for aromatic biosynthesis. *Pyrococcus horikoshii* has experienced total reductive evolution. Only TrpEb remains in *P. horikoshii*, and the case has been made that this may have some other function, such as serine deaminase activity (92). *P. abyssi* possesses genes encoding common-pathway and Trp pathway steps but lacks the Phe and Tyr branches. Although chorismate mutase (*aroQ*) is present, it could have some other substrate specificity (13). Since *P. abyssi* lacks the competing Phe and Tyr branches, an unusual metabolic circumstance exists in which the representation of tryptophan biosynthesis can be collapsed to that of a linear pathway of 12 overall steps (corresponding to the seven common-pathway steps followed by the five overall steps that are specifically dedicated to Trp biosynthesis). In contrast to the foregoing two differentially auxotrophic species of *Pyrococcus*, *P. furiosus* possesses a complete assemblage of aromatic-pathway genes.

Organisms that lack the entire branched system of aromatic amino acid biosynthesis include *P. horikoshii* (Fig. 2A), *Ureaplasma urealyticum* and *Mycoplasma* species (Fig. 2B), *Borrelia burgdorferi* and *Treponema pallidum* (Fig. 2C), and *Rickettsia prowazekii* and *Wolbachia* spp. (Fig. 2D). These whole-pathway reductive evolutions are generally associated with intracellular parasitism or endosymbiosis, and they imply auxotrophic dependence upon the host not only for all three aromatic amino acids but also for end products of the vitamin-like branches (e.g., folate, vitamin K, and ubiquinones) that derive from chorismate. In the *Bacteria*, some organisms possess an otherwise intact aromatic pathway but the Trp branch is uniquely absent. Among gram-positive bacteria (Fig. 2B), this includes *Enterococcus faecalis* and *Clostridium difficile*, and this pattern is also seen in the gram-negative *Haemophilus ducreyi* (Fig. 2D).

Interestingly, some organisms lack all three of the terminal aromatic amino acid branches but possess an intact common pathway to chorismate: *Streptococcus pyogenes* (Fig. 2B), *Streptococcus equi* (Fig. 1B), chlamydial species (Fig. 2C), *Porphyromonas gingivalis* (Fig. 2C), and *Treponema denticola* (Fig. 2C). The implication is that the remaining common pathway still links to one or more of the vitamin-like pathways. In the chlamydiae, we could not detect (by use of homology searching) a single gene encoding any known chorismate-utilizing enzyme. However, this could easily be accounted for by the existence of analogue genes that have not yet been identified. For example, *E. coli* chorismate lyase, which catalyzes the initial step of ubiquinone biosynthesis, is encoded by a gene (66) that is of very limited distribution. Therefore, elucidation of presently unknown analogue genes encoding chorismate lyase surely must be forthcoming.

Search for an Elusive *trpC* Gene in Actinomycete Bacteria

A particularly challenging observation was that, aside from the fragmented presence of the Trp pathway genes already discussed for the chlamydiae, some organisms lacked a single gene of Trp biosynthesis (*trpC* in all cases). These organisms are restricted to a cohesive cluster of gram-positive actinomycete bacteria (Fig. 2B and Fig. 3) that includes *Thermonospora fusca*, *Streptomyces coelicolor*, *Corynebacterium diphtheriae*, *Corynebacterium glutamicum*, and five species of *Mycobacterium*. Since *S. coelicolor* can grow on defined minimal medium in the absence of Trp, it must possess an intact Trp pathway. Likewise, *Mycobacterium smegmatis* is a saprophytic species that can grow in a minimal medium in the absence of Trp. This therefore also indicates the presence of a functional pathway even though the presence of *trpC* in the genome is not apparent by homology searching.

One actinomycete exception is explained by LGT. Within the actinomycete clade, the two species of *Corynebacterium* do possess a recognizable *trpC* (albeit fused to the *trpD* domain). However, this exception is explained by LGT displacement of not only *trpC* but also all *trp* genes in the *Corynebacterium* genus (except for a now-redundant *trpD* remnant) by the whole-pathway operon originating from an enteric bacterium (Xie and Jensen, unpublished data). Figure 3 shows that this actinomycete clade characteristically possesses a partial-pathway operon, *trpAa*/*trpD*/*trpEb*/*trpEa*, with gene insertions expanding the intergenic space between *trpAa* and *trpD*. In *T.*

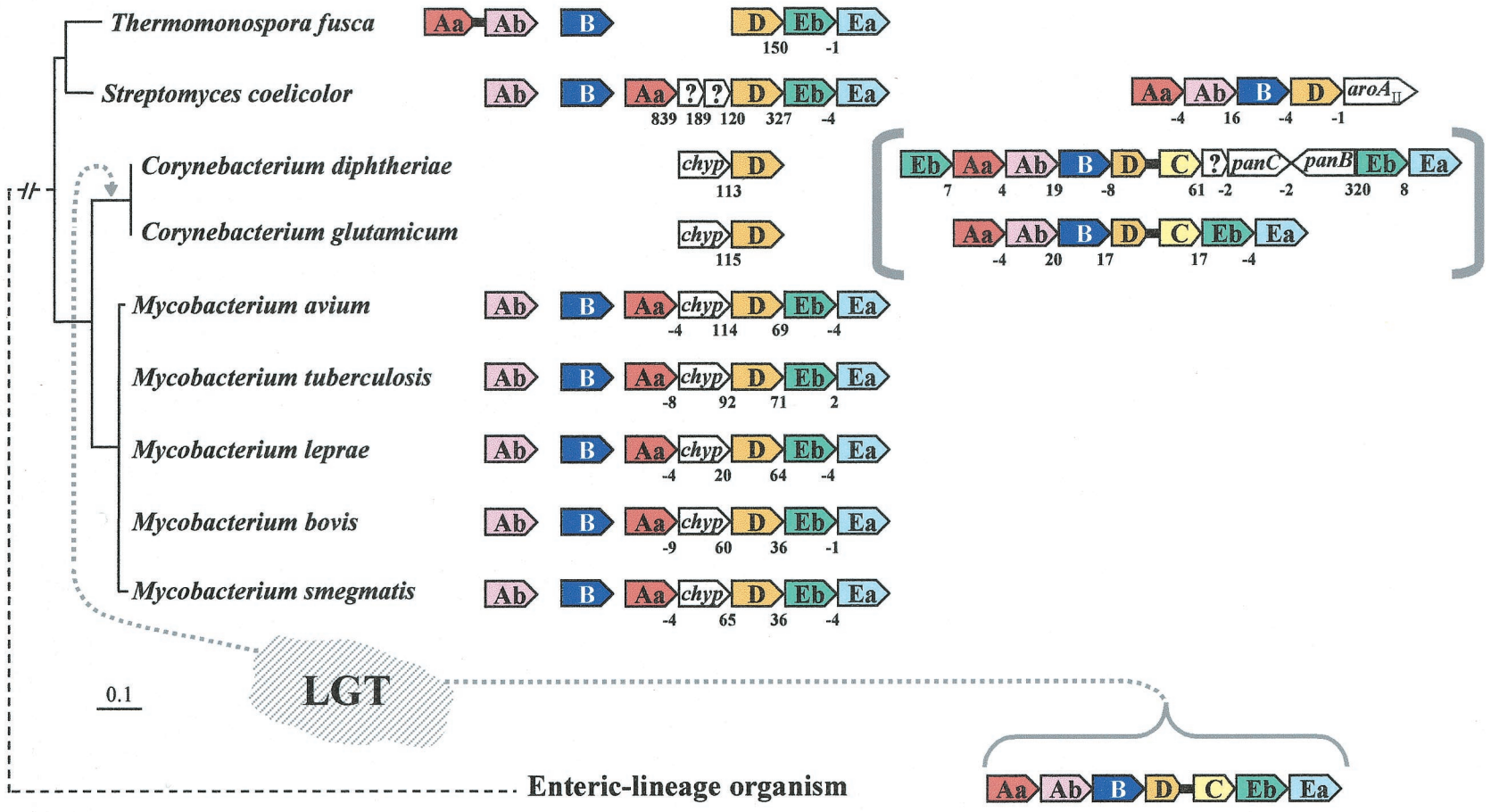


FIG. 3. Apparent absence of *trpC* and an event of LGT in a lineage of actinomycete bacteria. A broader phylogenetic context can be viewed in Fig. 2B and 6A. *chyp* denotes a conserved hypothetical membrane protein exhibiting about 28% identity in comparison of a given *Mycobacterium* species with a given *Corynebacterium* species. Color-coded boxes pointing in the direction of transcription represent genes of Trp biosynthesis. For clarity of presentation, *trpAa* is shown as *Aa*, etc. Open boxes with question marks denote hypothetical proteins. Intergenic spacing is shown, with negative values indicating gene overlap. *trpD●trpC* fusions are represented by short black linker bars. On the left are 16S rRNA-based phylogenetic trees of the genomes having the gene organizations shown on the right. Orthologues that match the mycobacterial *trpAa/chyp/D/Eb/Ea* operon genes are aligned vertically. Contemporary *trp* operons in coryneform species that originated in their common ancestor by LGT of *trpAa/Ab/B/D●C/Eb/Ea* from a source within the enteric lineage are shown within brackets. Except for the two coryneform species, all actinomycetes have a free-standing *trpB* gene. The *Mycobacterium* spp. and *Streptomyces* also have a free-standing *trpAb* gene. The corresponding TrpB and TrpAb proteins exhibit high identity with one another but not with TrpB and TrpAb of the coryneform species. *Thermomonospora* has dissociated *trpAa* from the typical clade operon and fused it with *trpAb* (as also shown in Fig. 4). The *trpAa/Ab/B/D/aroA*$_{II}$ operon of *S. coelicolor* is known to be specifically associated with antibiotic biosynthesis (see text).

fusca, *trpAa* has not only dissociated completely from the *trpD*/*Eb*/*Ea* operon but has fused with *trpAb*. Only *trpD* and an associated conserved hypothetical gene denoted *chyp* remain in species of *Corynebacterium* as remnants of the original actinomycete genes. The remnants are pleasingly fortuitous because they show the *Corynebacterium* ancestor to be the recipient of LGT rather than the donor.

A comprehensive phylogenetic tree for *trpD* proteins (data not shown) reveals that all of the TrpD proteins in Fig. 3 exhibit cohesive clustering and an order of branching that is congruent with the corresponding genome positions on the16S rRNA phylogenetic tree except, of course, for the *trpD* domain of the *trpD*•*trpC* fusion protein in the two coryneform species. Thus, in *C. diphtheriae* and *C. glutamicum*, the free-standing *trpD* outside of the whole-pathway *trp* operon is more closely related to *trpD* inside the partial-pathway *trp* operons of all the other organisms. An inner-membrane protein of unknown function separating *trpAa* and *trpD* in all of the mycobacteria, encoded by *chyp*, also flanks the nonoperonic *trpD* of the two coryneform species. As expected for the suggested LGT scenario, trees of TrpAa, TrpEa, and TrpEb proteins that are encoded from the partial-pathway operons of mycobacterial species, *Streptomyces*, and *Thermomonospora* in Fig. 3 all cluster closely together with the exclusion of the corresponding LGT genes from the coryneform bacteria.

Post-LGT events of vertical descent can be tracked in *C. diphtheriae*. Since the time that an alien *trpAa*/*trpAb*/*trpB*/*trpD*•*trpC*/*trpEb*/*trpEa* operon displaced the *trp* genes present in the common ancestor of coryneform bacteria, leaving behind only *chyp* and *trpD* as remnants, subsequent vertical evolutionary events in the *C. diphtheriae* genome are apparent. Thus, an insertion containing *panB* and *panC* occurred recently between *trpD*•*trpC* and *trpEb* in the *C. diphtheriae* lineage after its divergence from *C. glutamicum*. In *C. glutamicum*, closely related *panB and panC* orthologues (encoding ketopantoate hydroxymethyltransferase and pantothenate synthetase) comprise a characterized operon of D-pantothenate biosynthesis that is located elsewhere in the genome (71). In *C. diphtheriae*, the translocation of *panB* and *panC* into the *trp* operon is associated with an inversion event between these two genes. Hence, the opposite transcriptional direction of the inserted *panC* has now isolated *trpEb*/*trpEa* from its former operonic transcriptional continuity, presumably forcing it to become a separate transcriptional unit. It is interesting that the otherwise alien operon of *C. diphtheriae* now contains the native genes *panB* and *panC*, transposed from the resident genome. *C. diphtheriae* has also produced a gene duplicate of the gene encoding the alien TrpEb, which has then become the proximal member of the operon. This paralogue TrpEb is probably deficient in complex formation with TrpEa, because conserved residue K-167 (*Salmonella enterica* serovar Typhimurium numbering), which forms a salt bridge with residue D-56 of TrpEa, has been changed to S-167 (85). Also, the highly conserved residue 162-G has been changed to a charged residue, 162-E. Thus, after the LGT event, several subsequent vertical events of evolution that occurred in *C. diphtheriae* but not in *C. glutamicum* can be tracked.

The following approaches were taken in an attempt to locate the missing *trpC* genes in the above-mentioned actinomycete organisms.

Pattern and profile search. TrpC is a short and relatively divergent sequence. Known TrpC homologues may have identities as low as 22%. In an initial Blast screening with *E. coli* TrpC as the query, for example, the *Ferroplasma acidarmanus* genome did not return any hits and appeared to lack TrpC by this criterion. However, the position of an unknown gene within the *trp* operon of *F. acidarmanus* strongly implicated its presence as a divergent *trpC* gene because it occupies the same relative position as *trpC* in two closely related *Thermoplasma* species. Indeed, identity as *trpC* (second iteration) was amply confirmed by use of PSI-Blast (5), as well as by the observed conservation in multiple alignments of critical residues established by structural studies of TrpC from *E. coli*. In addition, the use of TrpC query sequences from most of the *Archaea* did return positive Blast hits from the *F. acidarmanus* genome.

With this background in mind, the genomes of *T. fusca*, *S. coelicolor*, and the mycobacteria *M. avium*, *M. tuberculosis*, and *M. bovis* were subjected to a pattern and profile search that included a ProSite-like pattern based upon critical residues reported in the PDB summary, the use of TrpC domains as query sequences that were available from the closest relatives of the group missing TrpC, and the generation of a hidden Markov model based on a multiple sequence alignment of known TrpC sequences. No illuminating results were obtained with this approach.

Evaluation of an unknown gene inserted in the *trp* operon. *M. tuberculosis* has a conserved hypothetical gene (Rv1610) inserted between *trpAa* and *trpD* (denoted *chyp* in Fig. 3). The absence of *trpC* coupled with the insertion of this unexpected gene within the *trp* operon invited careful scrutiny. This was, in fact, reminiscent of the previously mentioned situation with the operonic *trpC* of *F. acidarmanus*, which initially eluded detection as *trpC*. However, critical residues expected of TrpC could not be matched to Rv1610 by manual alignment. Furthermore, Rv1610 appears to encode an inner-membrane protein with three transmembrane segments. In addition, if Rv1610 were, in fact, a divergent TrpC, we would expect to find homologues in *T. fusca* and *S. coelicolor*. We did not.

Possible catalysis of the TrpC reaction by HisA. TrpC catalyzes an intramolecular oxidoreduction (Amadori rearrangement) that parallels the isomerase reaction catalyzed by HisA. Both reactions involve isomerization of an identical phosphoribosyl moiety. TrpC and HisA each exhibit $(\beta\alpha)8$ barrel structures. Jurgens et al. (46) in fact generated *hisA* mutants that could catalyze the TrpC reaction both in vivo and in vitro. One of these variants retained significant HisA activity. We therefore envisioned the possibility that an ancestor of the TrpC-deficient block of organisms might have duplicated *hisA* and recruited one copy to TrpC function. However, second copies of *hisA* were not found. We then further considered the possibility that HisA in these organisms might catalyze both reactions, since that potential had been established in vitro. However, the alignment of HisA sequences did not reveal any obvious variant residues common to the TrpC-deficient block of organisms that might suggest potential for TrpC activity.

Evolution of competence for TrpC catalysis by TrpD. Altamirano et al. (3) recently reported the evolution of TrpC activity from the $\alpha\beta$ barrel scaffold of TrpD following in vitro mutagenesis and recombination. Thus, one might envision an event of *trpD* gene duplication followed by divergence of one

of the paralogues to TrpC function. Although a gene duplicate of *trpD* was found in *S. coelicolor*, other organisms of the *trpC*-"deficient" block do not have a *trpD* gene duplicate. In consideration of the additional possibility that a modified *trpD* might encode an enzyme capable of both reactions, a careful comparison of the multiple alignment for *trpD* sequences failed to reveal a variant subgroup that might be expected of an evolved dual-function *trpC*/*trpD* protein. This is perhaps not surprising in view of the recent retraction (4) of the results of Altamirano et al. (3).

Other possibilities. Enzymes possessing triose phosphate isomerase (TIM) $(\beta\alpha)_8$ barrel-like folds are widespread and accommodate a particularly wide range of functions (15). Within this large grouping, TrpC, TrpD, TrpAa, and Rpe (D-ribulose 5-phosphate 3-phosphate epimerase) belong to the ribulose phosphate binding superfamily within the SCOP (structural classification of proteins) database (15, 86). Therefore, both TrpAa and Rpe were also evaluated as possible evolutionary sources of the missing TrpC, with the approaches described for HisA and TrpD. Suggestive evidence was not found.

The isomerase step catalyzed by TrpC is clearly a facile reaction, and although none of the foregoing possibilities considered produced the answer sought, they illustrate nicely the rationale and sorts of in silico strategies for gene discovery that can be anticipated in the near future. Until the time that this article was under review, the identity of *trpC* in the organisms included in Fig. 3 had remained a mystery. However, convincing evidence has been obtained recently that the HisA isomerase in these organisms does in fact catalyze the isomerase reaction in both pathways (9). The gene name, *priA* (phosphoribosyl transferase A), has been suggested to accommodate to its functional role in two pathways. Although this possibility was anticipated as outlined earlier, the natural bifunctional proteins of actinomycete bacteria did not resemble that obtained experimentally (46) in terms of amino acid sequence matches. Barona-Gómez and Hodgson (9) suggested that the bifunctional actinomycete isomerases represent an ancient evolutionary state that is in line with the recruitment hypothesis (38). If so, specialization in the gene duplicate that became *trpC* must have required more divergence than the gene duplicate that became *hisA* because the homology of PriA proteins with HisA is evident but not with TrpC proteins.

GENE FUSIONS

Phylogenetic Distribution of *trp* Gene Fusions

Each of the *trp* genes has been involved in various prokaryote fusion events except for *trpE*a. In some eukaryotes, however, trpEa and trpEb are fused (12, 16). Indeed, in *Euglena*, all of the *trp* genes except for *trpAa* and *trpAb* are fused together to form a pentafunctional protein (74). A *trpD●trpB* fusion is known in only a single instance (*Archaeoglobus fulgidus*), and a *trpC●trpEb* fusion is also thus far known in a single case (*Coxiella burnetii*). The remaining fusion types, all in the *Bacteria*, show an erratic distribution that is phylogenetically incongruous when mapped on the 16S rRNA tree (Fig. 4). Thus, the *trpAb●trpB* fusion is present not only in a small subcluster of the enteric bacteria, but also in the remote taxa *Thermotoga maritima and Campylobacter jejuni*. The *trpD●trpC* fusion, present throughout most of the enteric lineage (gamma proteobacteria), is also present in the widely separated *Helicobacter pylori* and in species of *Corynebacterium*. (In this case, we have already mentioned that a single origin followed by LGT events is likely.) Two distinct types of *trpAa●trpAb* fusions have occurred, one dedicated to primary biosynthesis (denoted *trpAa●trpAb*) and the other to phenazine pigment synthesis (denoted *trpAa●trpAb_phz*). The considerable extent of amino acid changes in TrpAa●TrpAb_phz has resulted in a shortened protein which no longer allows the ADIC product to continue through the ADIC lyase reaction to yield anthranilate, as is the case with anthranilate synthase (Fig. 1).

TABLE 3. Comparison of GC content in gene fusions and cognate genomes

Fusion	Organism	GI no.[a]	% G+C	
			Gene fusion	Genome
trpAb●trpB	*Escherichia coli*	2506459	56	51
	Salmonella enterica	1351306	57	55
	Thermotoga maritima	6226721	50	47
	Campylobacter jejuni	11268541	31	31
trpD●trpB	*Archaeoglobus fulgidus*	11499197	52	49
trpD●trpC	*Escherichia coli*	136292	53	51
	Vibrio cholerae	9655647	50	46
	Haemophilus influenzae	1574224	39	39
	Buchnera sp.	10038954	24	27
	Pasteurella multocida	12720848	41	38
	Helicobacter pylori	7227935	37	40
	Salmonella enterica	136301	55	55
	Vibrio parahaemolyticus	136302	47	46
	Shewanella putrefaciens	N/A	48	46
	Yersinia pestis	16122433	51	50
	Corynebacterium diphtheriae	N/A	57	53
	Corynebacterium diphtheriae	136291	58	56
trpAa●trpAb	*Brucella melitensis*	13487153	58	56
	Rhizobium meliloti	136328	63	62
	Azospirillum brasilense	1717765	74	68
	Anabaena sp.	17227910	41	44
	Anabaena sp.	17230725	46	44
	Nostoc punctiforme	N/A	42	44
	Thermomonospora fusca	N/A	68	69
	Rhodopseudomonas palustris	N/A	67	65
	Mesorhizobium loti	13472468	64	57
	Legionella pneumophila	N/A	38	40
	Agrobacterium tumefaciens	15889565	60	60
trpAa●trpAb_phz	*Streptomyces coelicolor*	21220595	75	72
	Pseudomonas aeruginosa	15597100	70	67
	Streptomyces violaceus	7481909	74	70
	Pseudomonas chlororaphis	6572982	63	62
	Pseudomonas fluorescens	2494756	62	62
	Pseudomonas aureofaciens	2494755	63	62

[a] GI, gene identification; N/A, not available.

A priori, the scattered phylogenetic distribution of these gene fusions could be attributed to (i) LGT, (ii) an initial ancestral fusion (of rare occurrence) followed by numerous events of gene loss in different lineages, or (iii) independent gene fusions (therefore being of relatively frequent occurrence). Table 3 shows that all of the gene fusions exhibit a GC content that is similar to that of the resident genome. Thus, either these did not originate by LGT, the donor genome fortuitously had a similar GC content, or the LGT event occurred sufficiently long ago that amelioration has masked LGT. Unpublished data

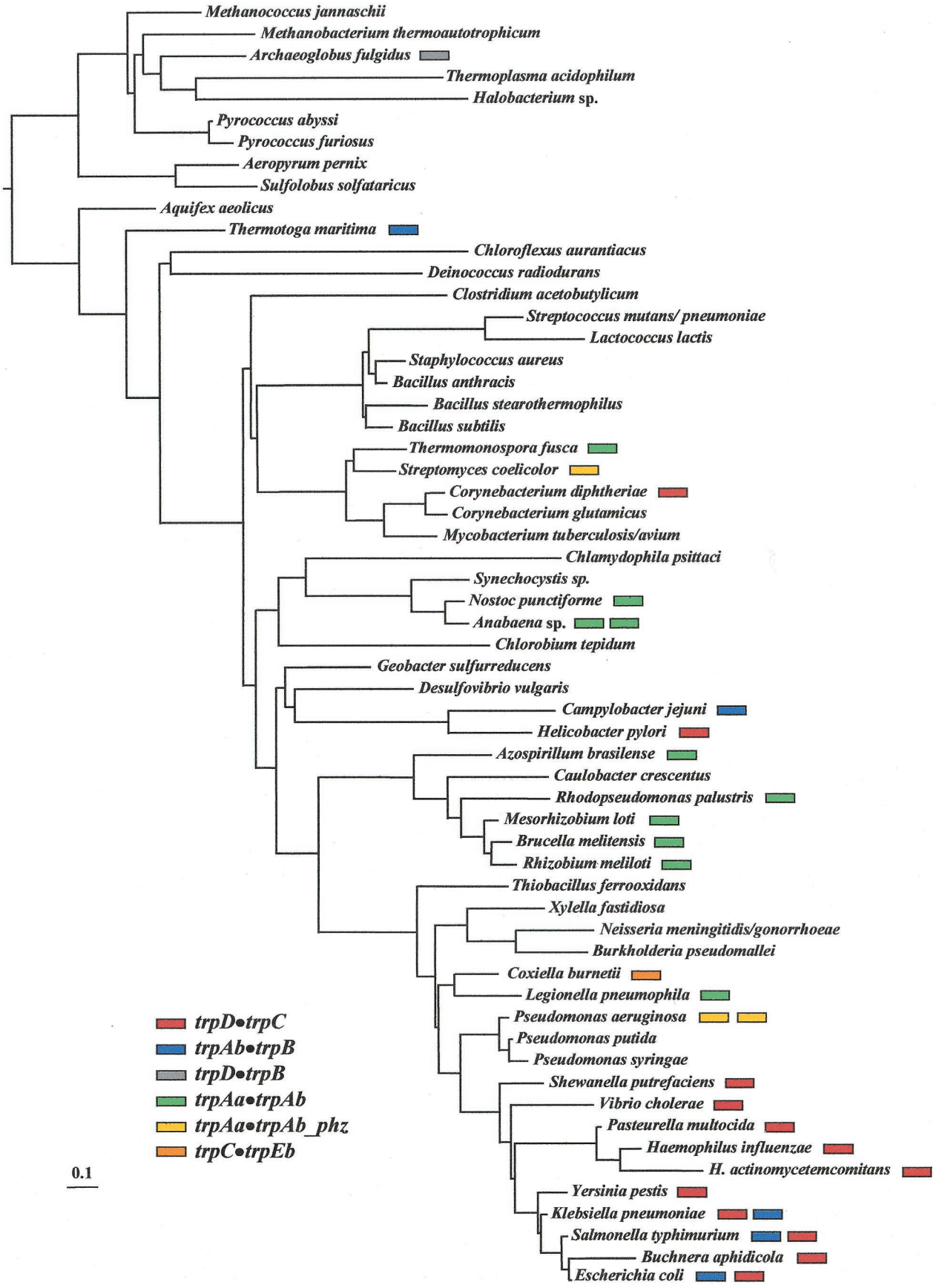


FIG. 4. Mapping of the distribution of Trp pathway gene fusions to the 16S rRNA tree. The presence of fusion subtypes is color-coded as indicated in the legend. Although *Buchnera aphidicola* maps near *E. coli* on the 16S rRNA tree, as shown, its true point of divergence is probably prior to *Yersinia*, as portrayed by dotted lines in Fig. 8.

(Xie and Jensen, unpublished data) support the occurrence of many of the gene fusions as independent events of evolutionary innovation. Although the *trpD●trpC* fusions in coryneform bacteria and in *Helicobacter pylori* originated from the enteric lineage by LGT, the comparison of parametric data, e.g., GC content, does not reflect this, probably due to amelioration.

Nested Gene Fusions

Jensen and Ahmad (1, 41) proposed that a series of nested gene fusions could be exploited as markers of phylogenetic branch points in prokaryotes. Thus, any organism that belongs to the enteric lineage (shaded green in Fig. 2D) shown in Fig. 4 would be expected to possess the *trpD●trpC* fusion, provided that the pathway has not been lost. At a more narrow hierarchical level, any organism belonging to the *E. coli*/*S. enterica* serovar Typhimurium/*Klebsiella pneumoniae* clade would be expected to possess the *trpAb●trpB* fusion as well. Thus, the clade defined by the *trpAb●trpB* fusion is nested within the more ancient clade defined by the *trpD●trpC* fusion. The presence of an AroQ●AroA$_{I\beta}$ (chorismate mutase●DAHP synthase) fusion in *Listeria*, *Bacillus* species, and *Staphylococcus* but not in *Enterococcus*, *Streptococcus*, or *Lactococcus* is consistent with the suggestion made earlier that, contrary to the 16S rRNA tree, the order of branching is slightly different, so that these group diverged at a deeper tree position.

The ultimate analysis of the total inventory of fused genes in any given genome should provide an excellent phylogenetic tool for deducing the order of branching. This approach should be greatly enhanced by the rapid increase in the number of sequenced genomes coupled with the enormous advantage of being able to identify gene fusions with bioinformatic methods. However, it was not expected at the time that fusions could occur independently at such frequencies or that LGT should be taken seriously. Therefore, application of the approach of nested gene fusions will require sufficient background work to recognize and discriminate fusion clusters that have independent origins on the vertical tree as well as ones that might have been spread in the horizontal direction by LGT.

Trp PATHWAY GENE ORGANIZATION IN THE *ARCHAEA*

In general the *Archaea* deploy the Trp pathway genes as whole-pathway operons or as partial-pathway operons (Fig. 5). A very limited amount of experimental work provides data supporting the qualitative existence of regulation at the transcriptional level (26, 77). Rearrangements of gene order following events of inversion, translocation, and gene loss have been sufficiently dynamic that it is currently not possible to deduce the gene order of the common ancestor without more closely spaced genome representation. The only certainty would appear to be the existence in the archaeal ancestor of the partial gene orders *→trpAa→trpAb* and *→ trpEb_1→trpEa*.

In the compact *Pyrococcus* genus, *P. horikoshii* has lost the entire pathway. Although the *trp* operons of *P. abyssi* and *P. furiosus* are virtually identical, great variation can be seen for the remainder of aromatic biosynthesis (see Fig. 2A). In the *Crenarchaeota* grouping (*Pyrobaculum*, *Aeropyrum*, and *Sulfolobus*; lowest clade of Fig. 5), dramatic scrambling of gene order is apparent. This group has replaced *trpEb_1* with *trpEb_2*. *trpEb_2* is a distinct subgroup of *trpEb* that is present mainly in *Archaea* and that may often (but not always, as indeed exemplified by the Crenarchaeota) have a separate stand-alone function (92).

Usually, the pair of genes encoding the two subunits of tryptophan synthase are adjacent in prokaryotes. In the case of *P. aerophilum*, *trpEa* and *trpEb_2* have been separated from one another within the operon. This may reflect the inability of *trpEb_2* to form a complex with *trpEa*. In *P. aerophilum*, *trpC* and *trpD* have become separately dissociated from the operon. *trpC* and *trpD* are adjacent in the operon of *Aeropyrum pernix*, but separated in the operon of *Sulfolobus solfataricus*. Although all of the *trp* genes in *A. pernix* are adjacent, they are organized as two divergently transcribed groups, *trpEa*/*trpEb_2*/*trpC*/*trpD* and *trpB*/*trpAa*/*trpAb*. The *A. pernix →trpEa→trpEb* order is very unusual, the *→trpEb→trpEa* gene order being one of the most highly conserved gene couples in all prokaryotic genomes (17). *Methanosarcina barkeri* and *Halobacterium* spp. have identical gene orders, but the intact operon currently seen in *M. barkeri* corresponds to a splitting into two separate operons in *Halobacterium* spp. In some cases, other aromatic-pathway genes have been inserted into the *trp* operon. Thus, the *trp* operon of *F. acidarmanus* has *aroA*$_{I\beta}$ as its most distal gene, whereas *S. solfataricus* has *aspC* (encoding aromatic aminotransferase) as the most distal gene of its *trp* operon.

Trp PATHWAY GENE ORGANIZATION IN THE *BACTERIA*

Whole-Pathway *trp* Operons

Unlike the domain *Archaea*, a consensus gene order can be discerned within the domain *Bacteria*, *trpAa*/*Ab*/*B*/*D*/*C*/*Eb*/*Ea* (Fig. 6A and 6B). The overall trace of organisms having the consensus gene order can be followed by noting the orange highlighting on the 16S rRNA tree of Fig. 2. A reasonable deduction is that this gene order (operon) was already present in the common ancestor of *Bacteria*, perhaps similar to the compact operons still present in the contemporary organisms *Thermotoga maritima*, *Listeria monocytogenes*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Coxiella burnetii.* In contrast to the compact operon structure of these organisms, where intergenic spacing is <70 nucleotides and indeed often exhibits negative values (gene overlap), other whole-pathway operons exhibit significantly greater intergenic spacing, sometimes in the form of insertions of seemingly irrelevant hypothetical genes. For example, although both *Lactococcus lactis* and *Cytophaga hutchinsonii* maintain a consensus operon gene order, *L. lactis* exhibits an intergenic space of 124 bp between *trpAa* and *trpAb*, whereas both *L. lactis* and *C. hutchinsonii* have inserted a hypothetical gene between *trpC* and *trpEb*. *Dehalococcoides ethenogenes* possesses a very compact but expanded operon that includes an insertion of *aroA*$_{I\beta}$ between *trpC* and *trpEb*. In this case, the insertion is probably physiologically relevant because AroA catalyzes the initial step of aromatic biosynthesis and thus forms the beginning precursor of chorismate.

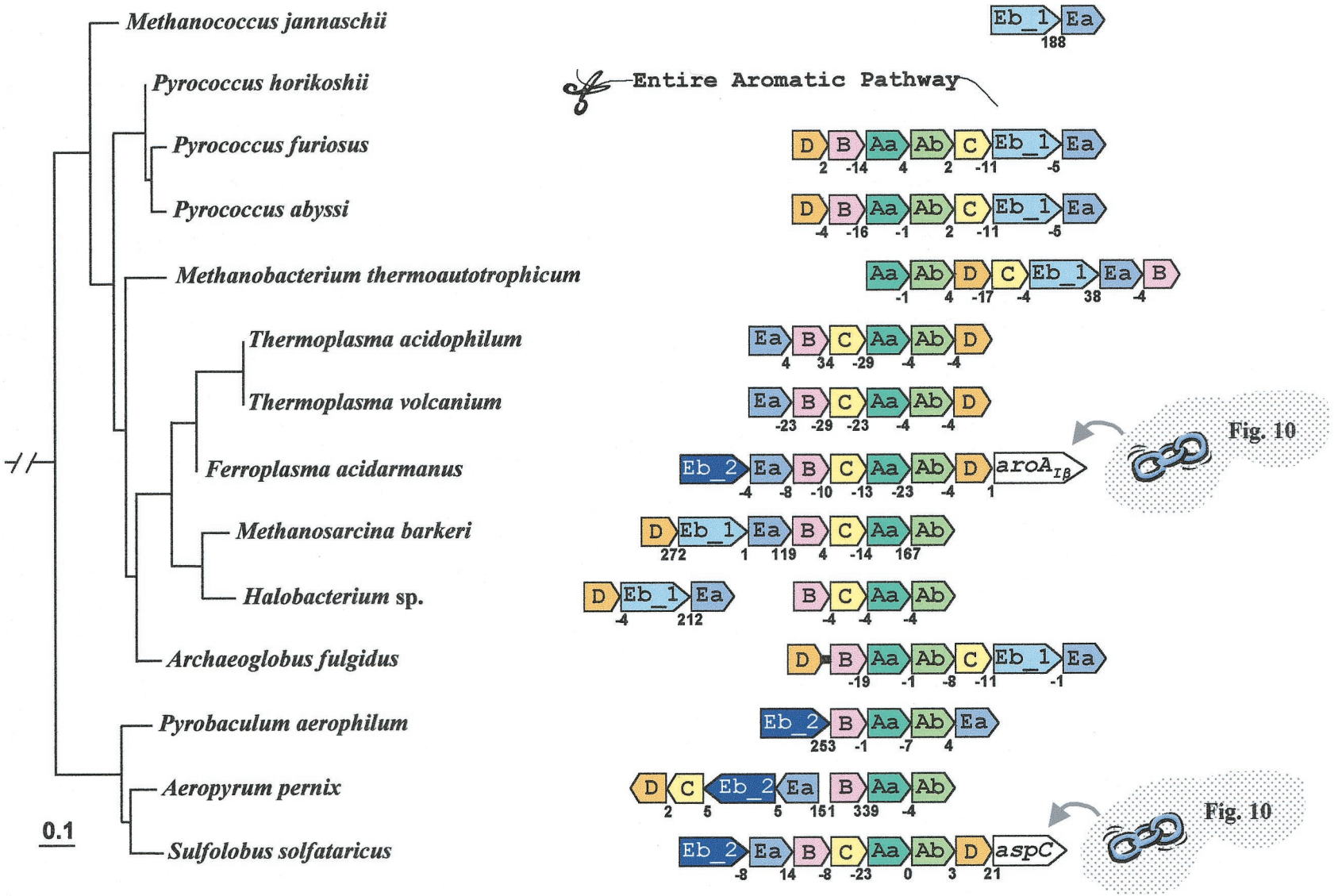


FIG. 5. Organization of *trp* operon genes in the *Archaea*. Each *trp* gene is color coded differently, including the two subtypes of *trpEb* (Eb_1 and Eb_2) (92). *trp* genes that exist in the genome unlinked to any other *trp* genes are not shown. *Archaeoglobus fulgidus* has a *trpD●trpB* gene fusion (see Fig. 4). Intergenic spacing is shown, with negative values indicating gene overlap. Genes that are not specific *trp* pathway genes are in white boxes. *F. acidarmanus* possesses a gene encoding the *aroA*$_I\beta$ subclass (44) of DAHP synthase. *aspC* in *S. solfataricus* is an aromatic aminotransferase of the Iγ aspartate aminotransferase type (42). This gene insertion corresponds to genes that appear to have escaped from the *aro* operons shown in Fig. 10. The gene order shown for *Methanosarcina barkeri* is the same as those in *Methanosarcina acetivorans* and *Methanosarcina mazei*. The gene order shown for *S. solfataricus* is the same as that for *Solfolobus tokodaii*.

Dispersal of *trp* Operon Genes

Gene dissociation has disrupted the *trp* operon of *Bacteria* in occasional lineages. For example, as illustrated at the bottom of Fig. 6A, *Campylobacter jejuni* possesses an extremely compact *trp* operon, but *trpD* has become dissociated, leaving behind a six-member partial-pathway *trp* operon. In *Deinococcus radiodurans* the separate dissociations of *trpAa*, *trpD*, and *trpC* have resulted in the retention of two small operons (each with overlapping genes) that are remnants of the ancestral whole-pathway operon. In these figures, isolated *trp* genes are not shown in order to conserve space, but they are present in the genome unless their absence is indicated in Fig. 2, e.g., *trpAa* and *trpAb* are missing in species of *Chlamydophila psittaci* (Fig. 2C).

In some cases, bacteria possess two chromosomes (19, 54). It is interesting that, in *Rhodobacter sphaeroides*, not only has the ancestral *trp* operon been split apart, but also the resulting partial-pathway operons (*trpAa*/*yibQ*/*trpAb*/*trpB*/*trpD* and *trpC*/*aroR*/*trpEb*) now reside on separate chromosomes. TrpEa has become completely dissociated from these operons (54). The closest available genomic neighbor of *R. sphaeroides* that is available on the 16S rRNA tree is *Sphingomonas aromaticivorans*, and it possesses the same split-pathway arrangement as *R. sphaeroides* except that *trpEb* and *trpEa* have remained together (i.e., *trpC*/*trpEb*/*trpEa*). The intriguing partitioning of the *trp* split-pathway operons between two chromosomes is typical of a 16S rRNA grouping of organisms that includes *Rhodopseudomonas palustris*, *Rhizobium loti*, *Brucella melitensis*, *Sinorhizobium meliloti*, *Rhodobacter sphaeroides*, and *Sphingomonas aromaticivorans*. Most of these organisms are not shown in Fig. 6, but a detailed breakdown of Trp pathway gene organization in this part of the tree is given in reference 93.

At one extreme of gene dissociation, gene dispersal has completely eliminated any linkage of *trp* pathway genes, as observed in *Aquifex*, unicellular cyanobacteria (*Synechocystis*, *Synechococcus*, and *Prochlorococcus*), and *Chlorobium tepidum*. (Only organisms possessing at least some linked *trp* genes are shown in the various figures of this paper.) One might reasonably consider whether these organisms simply manifest retention of a "preoperon" ancestral state, but this seems untenable with respect to the application of parsimony principles because they represent distinctly separate, widely spaced lineages.

All cyanobacteria possess a common phylogenetically congruous set of completely dispersed genes for tryptophan biosynthesis. However, *Nostoc* and *Anabaena* possess in addition some redundant *trp* genes that are linked to one another. The assemblage of linked *trp* genes in *Anabaena* spp. (shown in the middle of Fig. 6A) is very similar to that of the closely related *Nostoc punctiforme* (not shown, but see Fig. 2 of reference 93 for details) and seems to be part of a larger gene assemblage (possible supraoperon) that includes several other aromatic-pathway genes. *Nostoc* and *Anabaena* (large-genome, filamentous, and heterocystous cyanobacteria) possess these linked genes in addition to copies of all of the dispersed *trp* pathway genes found in the unicellular cyanobacteria. Hence, the redundant set of linked genes that are uniquely present in *Nostoc* and *Anabaena* seemed to be obvious candidates for origin by LGT. However, no support for LGT was found, and it has been suggested (93) that ancient paralogues have been retained in the *Nostoc*/*Anabaena* lineage, whereas the set of linked paralogue genes has been lost in the unicellular cyanobacteria.

Gene Scrambling

Examples of extreme gene scrambling can be found in *Bacteria*, e.g., in *Desulfitobacterium hafniense* (middle of Fig. 6A). However, gene scrambling seems to have been generally less pronounced in the *Bacteria* than in the *Archaea*. One of the most bizarre *trp* operons of the *Bacteria* is that of *Geobacter sulfurreducens* (Fig. 6A). Not only have *trpEa* and *trpEb_1* dissociated from the operon and from one another, but *trpEb_2* (rarely found in *Bacteria*) has also been inserted into the operon between *trpD* and *trpC*. *trpEb_2* is an "alternative" β subunit of tryptophan synthase whose usual functional role has been speculated to be catalysis of the serine deaminase reaction (92). The gene fusions designated by short connecting black bars in Fig. 6A and Fig. 6B have already been discussed (Fig. 4).

Brown and Doolittle (11) made the correct observations, as long ago as 1997, even with vastly less data, that the consensus gene order seemed to be *trpAa*/*Ab*/*B*/*D*/*C*/*Eb*/*Ea* in *Bacteria*, that archaeal gene orders seem to be more variable than in *Bacteria*, and that the *trpAa*/*trpAb* and *trpEb*/*trpEa* linkage groups might be ancestral.

RETENTION OF THE ANCESTRAL OPERON AT SPACED PHYLOGENETIC NODES IN *BACTERIA*

The gene order *trpAa*/*Ab*/*B*/*D*/*C*/*Eb*/*Ea* of *trp* operons generally persists in the phylogenetic section of *Bacteria* shown between *Thermotoga maritima* and *Helicobacter pylori* of Fig. 6A, and a rationale of parsimony supports the thesis that this operon was already present in the common ancestor of modern *Bacteria*. This is not immediately obvious to casual inspection because of the dynamics of gene dissociation, gene loss, gene scrambling, and gene dispersal. However, a progression of conserved ancestral operons can be identified from the deepest phylogenetic position to the point of operon splitting between *trpD* and *trpC* (see orange highlighting in Fig. 2).

At the deepest branching position shown in Fig. 6A, *T. maritima* possesses a compact ancestral operon, differing only in that *trpAb* and *trpB* have fused. This fusion is rare, having occurred elsewhere only in the distant *E. coli*/*S. enterica* serovar Typhimurium/*K. pneumoniae* subgrouping (Fig. 4). At the next phylogenetic node in Fig. 6A, *D. ethenogenes* has retained the ancestral *trp* operon, albeit with an $aroA_{I\beta}$ insertion between *trpC* and *trpEb*. In the gram-positive organisms shown in the Fig. 2B tree, ancestral operons are present in the following organisms from the deepest to more shallow phylogenetic nodes: *Clostridium acetobutylicum* > *Desulfitobacterium hafniense* > *Listeria monocytogenes*, *Bacillus anthracis*, and species of *Staphylococcus.* The ancestral *trp* operon has not survived in most of the phylogenetic groupings shown in Fig. 2C. In many cases, some or all of the Trp pathway genes have been lost by reductive evolution in pathogenic *Bacteria*. In other cases (cyanobacteria and *Chlorobium tepidum)*, the *trp* genes have all been dispersed. *Cytophaga hutchinsonii* is the sole organism

shown in Fig. 2C that has retained a complete *trp* operon with the ancestral gene order. Finally, in the top node illustrated in Fig. 2D, *Desulfovibrio vulgaris* has retained the compact ancestral *trp* operon, as shown near the bottom of Fig. 6A.

TWO MAJOR EVENTS UNDERLIE THE DYNAMICS OF *trp* OPERON CHANGE IN *BACTERIA*

Operon Scission Yields Two Half-Pathway Operons

In the common ancestor of the section of the 16S rRNA tree between *Caulobacter crescentus* and *Pseudomonas aeruginosa* (Fig. 6B), the ancestral operon was split into two, *trpAa*/*Ab*/*B*/*D* and *trpC*/*Eb*/*Ea*. The resulting two partial-pathway operons have exactly the gene organization as the contemporary *Thiobacillus ferooxidans, Xylella fastidiosa, and Methylococcus capsulatus*. An additional four genomes (*Caulobacter crescentus*, *Sphingomonas aromaticivorans*, *Ralstonia metallidurans*, and *Burkholderia fungorum*) exhibit the same two operons, but they are less compact. These partial-pathway operons are colored magenta in Fig. 2D. This split-operon pattern is very similar throughout this phylogenetic block of organisms, with some variations of gene insertion, gene dissociation, and gene fusion (see Fig. 9 in reference 93 for more detail).

Only *Magnetococcus* sp. and *Coxiella burnetii* in this section of the 16S rRNA tree exhibit *trpD* and *trpC* in contiguous positions. Presumably *trpD* and *trpC* were rejoined in these organisms. Zheng et al. (98) recently noted the frequency of split-pathway operons, and they made the intuitively reasonable conclusion that these are evolutionary forerunners of the complete *trp* operon. (In one sense, this is correct as a special case for the aforementioned *Coxiella burnetii.*) However, an inspection of the comprehensive set of data now available, with parsimonious principles applied, can only lead to the conclusion that a previously intact operon has undergone fragmentation.

Fusion of *trpD* with *trpC* Restores a Whole-Pathway Operon

In the common ancestor of the phylogenetic block of organisms sandwiched between *Shewanella putrefaciens* and *Escherichia coli* (Fig. 4, Fig. 6B, and Fig. 8), *trpD* and *trpC* were joined by fusion to restore an intact operon having the original ancestral gene order. All organisms within this "enteric" lineage possess the *trpD*●*trpC* fusion (green shading in Fig. 2D). Protein domain trees of the TrpD●TrpC proteins from the enteric lineage are highly congruent with 16S rRNA trees, as indeed are all seven protein domains. These protein trees (all seven) include out-of-position Trp proteins from *Helicobacter pylori*, *Corynebacterium glutamicum*, and *Corynebacterium diphtheriae*, the consequence of LGT (see next section).

It should be kept in mind that our perception of milestone evolutionary events is biased by the relatively unbalanced selection of complete genomes currently available, and this will undoubtedly be altered as genome representation in parts of the evolutionary tree that are currently sparsely represented expands and yields more balanced representation. Thus, we readily see splitting of the ancestral operon as a milestone event in the Proteobacteria because many proteobacterial genomes have been sequenced. Likewise, we see the fusion of the split-operon halves as another milestone event because organisms of the enteric lineage have received high priority for genome sequencing. In other words, the current biases in genome selection have favored the deduction of evolutionary events in those lineages. Thus, in the future one can expect an expansion of milestone events recognized in other lineages.

LATERAL GENE TRANSFER OF *trp* OPERONS

Lateral Gene Transfer of Whole-Pathway Operons

From the vantage point of primary Trp biosynthesis, free-living prokaryotes will already have a reasonably integrated Trp pathway, and it seems unlikely that any selective advantages would come from displacing the native pathway with an alien one which evolved in a different metabolic context. It is possible that organisms that have lost the Trp pathway might occasionally reacquire it in one LGT event (from a whole-operon pathway donor). One could envision displacement of a native operon by an alien one if these possessed a more effective regulation mechanism (provided that advanced regulation really meshes with the needs of the recipient). However, the most sophisticated regulatory systems thus far described seem to utilize unlinked regulatory genes, such as *trpR* in *E. coli* and *mtrB* in *B. subtilis*. Thus, it would be difficult to transfer the entire operon system of structural genes and one or more unlinked regulatory genes via LGT.

It also is worthwhile to consider whether what is effective regulation for one organism would be appropriate for organisms that have a completely different lifestyle. *E. coli*, for example, experiences regular episodes of feast and famine in the gut of humans, and the ability of *E. coli* to regulate Trp enzymes over a large range of expression confers rapid response and efficiency. On the other hand, cyanobacteria generally grow in a nutritionally dilute environment and synthesize most of their amino acids most of the time. Under these conditions, possession of an operon system that is responsive over several orders of magnitude may not confer selective advantages.

There are a number of well-spaced genomes that possess the putative ancestral operon of *Bacteria*, highlighted orange in Fig. 2, e.g., *trpAa*/*Ab*/*B*/*D*/*C*/*Eb*/*Ea* is present in species of *Listeria*, species of *Streptococcus*, species of *Staphylococcus*, *Clostridium acetobutylicum*, and *Desulfovibrio vulgaris*. We considered the possibility that the *trp* operons in these organisms are related to one another by LGT rather than by vertical descent. However, we did not find that the *trp* operon proteins in any of these organisms clustered together when comprehensive trees for all seven proteins were inspected (data not shown), as would be expected for relationships of LGT. Therefore, we conclude that in these lineages, the exact ancestral operon was simply retained without gene dispersal, gene insertion, or gene fusion.

On the other hand, the fusion-containing *trp* operon (*trpAa*/*Ab*/*B*/*D*/●*C*/*Eb*/*Ea*) in the enteric lineage is related to those of coryneform bacteria and *Helicobacter pylori* by LGT. We know that coryneform bacteria must have been the recipient rather than the donor because they retain remnants of the original host. We conclude that *H. pylori* was also a recipient of LGT from the ancestral lineage because the *Helicobacter*/*Campylobacter* node of divergence is more recent than the root of divergence for the enteric lineage. Therefore, if *Helicobacter* had

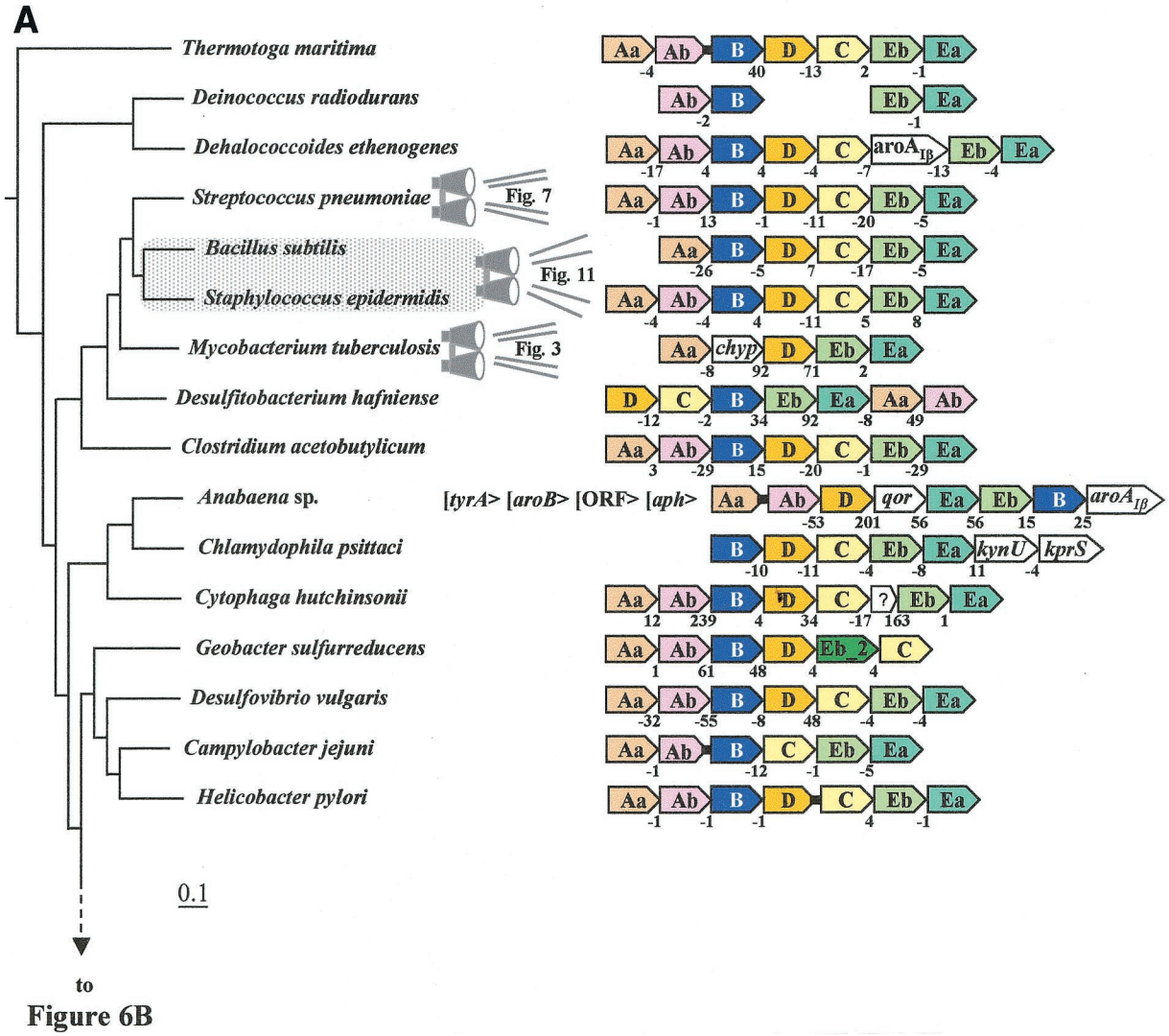


Figure 6B

FIG. 6. Organization of *trp* operon genes in the *Bacteria*. Each *trp* gene is color coded differently, including the two subtypes of *trpEb*. (*trpEb* in this figure refers to the major *trpEb*_1 subtype.) The tree sections in A and B join as indicated by the dashed line. Intergenic spacing is shown, with negative values indicating gene overlap. Separations showing white space and no intergenic spacing values indicate that the gene clusters are not linked to one another. Insertions of hypothetical genes and known genes are shown as white boxes. Short black bars connecting arrows denote gene fusions. Links to zoom-in expansions of particular lineages in other figures of this paper are indicated by binoculars. In B, the gene organization shown for *Rhodopseudomonas palustris* is identical to those of the closely related *Agrobacterium tumefaciens*, *Rhizobium loti*, *Brucella melitensis*, and *Sinorhizobium meliloti*; that shown for *Burkholderia fungorum* is identical to that of *Burkholderia pseudomallei* and *Burkholderia mallei*; that shown for *Bordetella parapertussis* is identical to that of *Bordetella pertusis* and *Bordetella bronchiseptica;* that for *Neisseria meningitidis* is identical to that of *Neisseria gonorrhoeae*; and that for *Pseudomonas aeruginosa* is identical to that of *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Pseudomonas syringae.* The apparent supraoperon of *Anabaena* sp. (A) has been discussed in reference 93. *kynU* and *kprS* on the *Chlamydophila psittaci* line (A) refer to genes encoding kynureninase and PRPP synthase, respectively (89). The linked *trpAa*/*trpAb* genes shown for *P. aeruginosa* (B) were named *phnA*/*phnB* by Essar et al. (24). because they were thought to be dedicated to phenazine biosynthesis, a conclusion shown to be incorrect by Mavrodi et al. (57). This gene pair is not within the vertical line of descent (see later section), as indicated by the LGT notation. The *trpAaAb* operon shown on the left for *Xylella* is also outside the vertical line of descent (i.e., origin by LGT) (93). *Shewanella putrefaciens* (B) has the newly proposed name of *Shewanella oneidensis* (81).

been the *trp* operon donor, one would expect *Campylobacter* to also have the fusion-containing *trp* operon. As pointed out before, the modern *Helicobacter* operon lacks repression control by *trpR*, presumably because *trpR* of the alien enteric lineage donor was unlinked to the transferred operon. It would be interesting to know how the regulation of the modern *H. pylori trp* operon compares to that of the modern *Campylobacter jejuni trp* operon, which presumably would be similar to the original *H. pylori trp* operon that was displaced.

Lateral Gene Ttransfer of Partial-Pathway *trp* Operons

Trp pathway enzymes can have metabolic roles other than to serve protein synthesis as a primary source of Trp. Specialized pathways leading to pigments, antibiotics, etc., have already been mentioned, and many unknown specialized pathways probably exist. Both partial-pathway and whole-pathway operons can be associated with specialized pathways. Cases in which one or more enzymes can serve the needs of both pri-

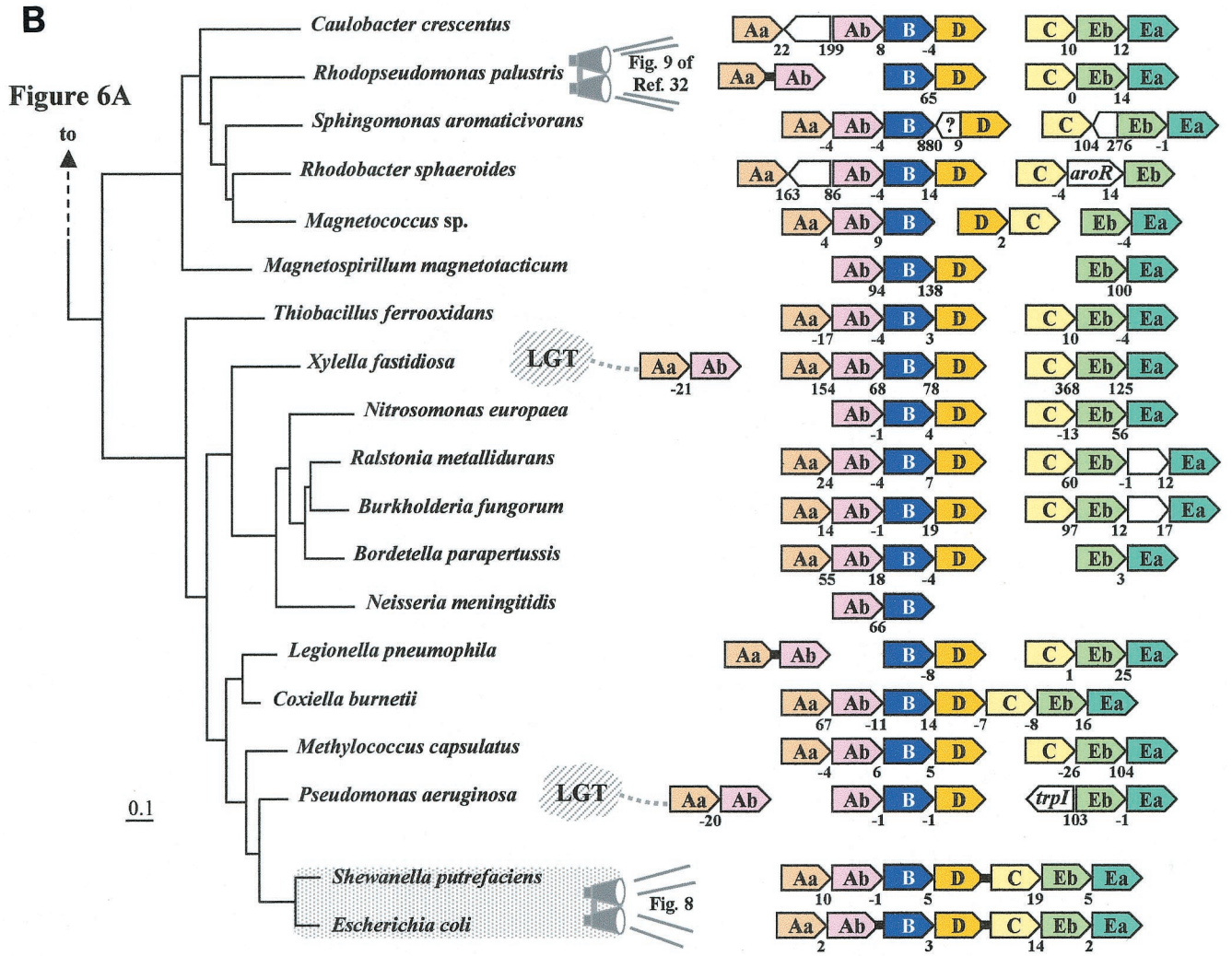


FIG. 6—*Continued.*

mary biosynthesis and some specialized pathway are known. It is quite common in such cases that the specialized pathway will possess key paralogues or analogues of Trp-regulated enzymes of the primary pathway. Such paralogues or analogues differ in the absence of the usual regulatory properties in order to abolish Trp as a regulatory cue (see below for examples). Operons encoding specialized pathways are more likely to confer immediate selective advantages to a recipient if a novel capability is transferred. Lawrence has asserted (51) that the selfish-operon model "predicts that operons are unstable as genes associate and disperse between transfer events." This scenario probably would be more applicable to *trp* operons associated with some metabolic specialization than to those associated with primary amino acid biosynthesis.

Figure 6B shows two different partial-pathway *trpAa*/*trpAb* operons that were acquired by LGT in *Xylella fastidiosa* and in *Pseudomonas aeruginosa*, as discussed previously by Xie et al. (93). In *Xylella* it has been speculated (93) that *trpAa*/*trpAb* coexists within an operon with *acl*, which encodes an aryl-coenzyme A ligase that might have the specificity of an anthranilate-coenzyme A ligase. This might then be a point of divergence, whereby coenzyme A-activated anthranilate proceeds to an antibiotic, siderophore, etc. This anthranilate synthase appears to be resistant to feedback inhibition by Trp, consistent with the absence of Trp as an end product of the putative specialized pathway. The *P. aeruginosa trpAa*/*trpAb* operon shown in Fig. 6B was originally denoted *phnA*/*phnB* (*phn* for phenazine) because their expression in stationary phase, unregulated by Trp, was thought to be a mechanism to produce anthranilate precursor for phenazine synthesis in the presence of Trp. Although it is now known (57) that this operon is not part of the phenazine pathway and that anthranilate is not a phenazine precursor, it would appear to constitute a system designed for production of anthranilate in an unknown functional role in stationary-phase metabolism.

Streptomyces coelicolor possesses an operon (*trpAa*/*trpab*/*trpB*/*trpD*/*aroA*$_{II}$) (Fig. 4) that is nested within a large cluster of genes that dictate synthesis of a calcium-dependent antibiotic (CDA) (70). This antibiotic contains Trp. The origin of this operon by LGT has been mentioned (70), but a detailed analysis has not yet been done. However, even if it originated via ancient paralogy instead, it is a good example of a contempo-

rary operon that could confer a specialized ability to make Trp in the presence of fully charged tryptophanyl-tRNA via LGT. The key aspects are an operon free of any mode of regulation by Trp and inclusion of the gene encoding a homologue of DAHP synthase ($AroA_{II}$) that is not inhibited by amino acids, hence ensuring an unrestrained supply of chorismate. Thus, normal restraints in place for primary biosynthesis at the branch point levels of both DAHP synthase and anthranilate synthase have been removed in order to accommodate the secondary synthesis of antibiotic. Note also in these examples with *S. coelicolor* that the primary and secondary pathways are not entirely separate.

The antibiotic-oriented operon system lacks *trpEa* and *trpEb*. Therefore, the tryptophan synthase that is utilized for primary biosynthesis must also be used to make Trp molecules destined for incorporation into antibiotic molecules. In view of the recent revelation (9) that *priA* fulfills the isomerase function in both the histidine and tryptophan pathways in *S. coelicolor*, as discussed earlier, it would appear that *priA* must also have a functional role in a third pathway to the CDA antibiotic. *S. coelicolor* has four paralogues of *trpAa*: one engaged in primary Trp biosynthesis (undoubtedly sensitive to feedback inhibition), a free-standing *trpAa* of unknown function, one dedicated to antibiotic biosynthesis (probably not sensitive to feedback inhibition), and another (not shown in Fig. 3) that is a domain component of *trpAa●trpAb_phz* and dedicated to phenazine biosynthesis.

FINE-TUNED EVOLUTIONARY DEDUCTIONS

At the exponentially increasing rate of genome sequencing, it is becoming feasible to examine, or at least to anticipate, the examination of organisms that are sufficiently close in a phylogenetic progression to facilitate refined evolutionary conclusions.

Single Change in a Common Ancestor versus Multiple Independent Changes in Descendants

Figure 7 illustrates the state of the *trp* operon in a gram-positive lineage containing *Listeria*, *Enterococcus*, *Streptococcus*, *Lactococcus*, *Ureaplasma*, and *Mycoplasma.* Some of these organisms have become auxotrophic following loss of the Trp branch (*Enterococcus*), loss of all three branches of aromatic biosynthesis (*Streptococcus pyogenes* and *Streptococcus equi*), or loss of the entire aromatic pathway (*Ureaplasma* and *Mycoplasma*). These events of gene loss had to be quite recent, undoubtedly linked to a relationship between the pathogenic lifestyles of these organisms and the relinquishing of selective pressure to retain the Trp pathway.

If the 16S rRNA tree (Fig. 2B) reflects an exactly correct order of branching, then reductive evolution led to the loss of the Trp pathway independently on four occasions: in *Enterococcus*, *S. pyogenes*, *S. equi*, and the common ancestor of the *Ureaplasma* and *Mycoplasma* genera. However, the resolution power of 16S rRNA for determination of exact branching order can be imperfect for closely related organisms, and other character states can help fine-tune branching orders. Figure 7 illustrates a suggested modification of the 16S rRNA branching order (as shown in Fig. 2B) so that *S. pyogenes* and *S. equi* have a common ancestor (Fig. 7), rather than a common ancestor for *S. equi* and *L. lactis* (Fig. 2B). This modified tree yields a parsimonious loss (one event) in the common ancestor of *S. pyogenes* and *S. equi*. Note that this alteration of branching order is conservative, considering that the distance between *S. pyogenes* and *S. equi* is distinctly less than the distance between *S. equi* and *L. lactis* on the16S rRNA tree of Fig. 2B.

Distinguishing Derived States from Ancestral States

Listeria and three species of the divergent *Streptococcus* genus have retained a compact operon of the ancestral order *trpAa*/*Ab*/*B*/*D*/*C*/*Eb*/*Ea.* In contrast, although *Lactococcus* exhibits gene overlap between gene pairs at three positions, expansion of intergenic spacing is evident between *trpAa* and *trpAb*, between *trpB* and *trpD*, and especially between *trpC* and *trpEb*. Evolutionary direction can be deduced; that is, it is more parsimonious to conclude that the intergenic expansions within the *L. lactis* operon are a derived state rather than the ancestral state. Other examples are given in the next two sections, and the ability to distinguish derived evolutionary states from ancestral states in sister lineages is key to the ability infer evolutionary character states at given phylogenetic nodes.

Deducing Ancestral Character States at Phylogenetic Node Positions

Figure 8 provides a zoom-in picture of the gene organization exhibited by the enteric lineage of *Bacteria* (gamma proteobacteria). This entire group had a common ancestor that acquired the landmark fusion of *trpD* and *trpC* (*trpD●trpC*), restoring a whole-pathway operon. In the *Pasteurella*/*Haemophilus* grouping, dynamic changes occurred, including both the separation of *trpEb*/trpEa from the original operon and expansion of the intergenic space between *trpAb* and *trpB* by insertion. After the divergence of *Pasteurella* from the common ancestor of the *Haemophilus* lineage, the entire assemblage of Trp pathway genes was discarded in *Haemophilus ducreyi.* On the other hand, in *Haemophilus actinomycetemcomitans*, the intergenic space between *trpB* and *trpD●trpC* was expanded by insertion of two hypothetical genes. In the lower cluster of organisms in Fig. 8, *trpAa*/*trpAb* became separated from the rest of the operon in the outlying lineage that is represented by *Buchnera* sp. In the common ancestor of *E. coli*, *S. enterica* serovar Typhimurium, and *Klebsiella pneumoniae*, a fusion between *trpAb* and *trpB* occurred at a very recent time. *trpAb●trpB* and *trpD●trpC* exemplify one of the sets of nested gene fusions discussed by Jensen and Ahmad (41) that can be exploited for hierarchical ordering of taxa.

Value of Flanking-Gene Context

Figure 9 shows the bacterial organisms in the section of the 16S rRNA tree whose common ancestor possessed the two split-pathway *trp* operons that resulted from the separation of the ancestral *trpAa*/*Ab*/*B*/*D*/*C*/*Eb*/*Ea* operon between *trpD* and *trpC*. These organisms include the α-Proteobacteria (top major grouping), the β-Proteobacteria, and some of the γ-Proteobacteria. We did not find the *trpAa*/*Ab*/*B*/*D* partial-pathway operon to be flanked by conserved genes, but the *trpC*/*Eb*/*Ea* par-

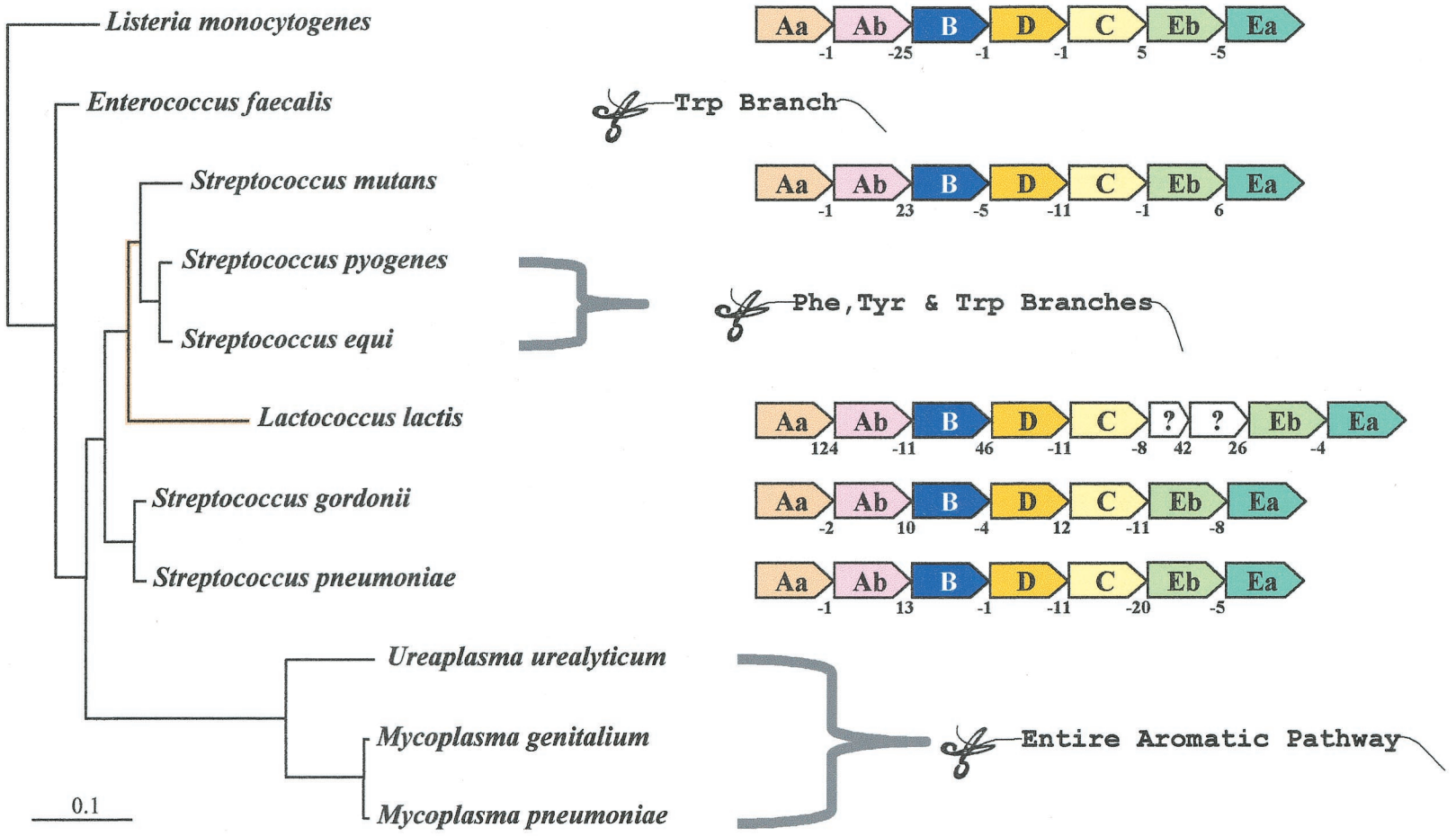


FIG. 7. Zoom-in from Fig. 6A showing instances of Trp pathway reductive evolution and expansion of intergenic space in one phylogenetic section of some gram-positive bacteria whose 16S rRNA tree relationships are shown at the left. Loss of various metabolic capabilities is indicated by scissors. Note that the order of branching of *Lactococcus lactis* (shown in orange) has been altered from that shown in the 16S rRNA tree of Fig. 2B. The gene order and compact spacing of *Listeria innocua* is the same as that shown for *Listeria monocytogenes*.

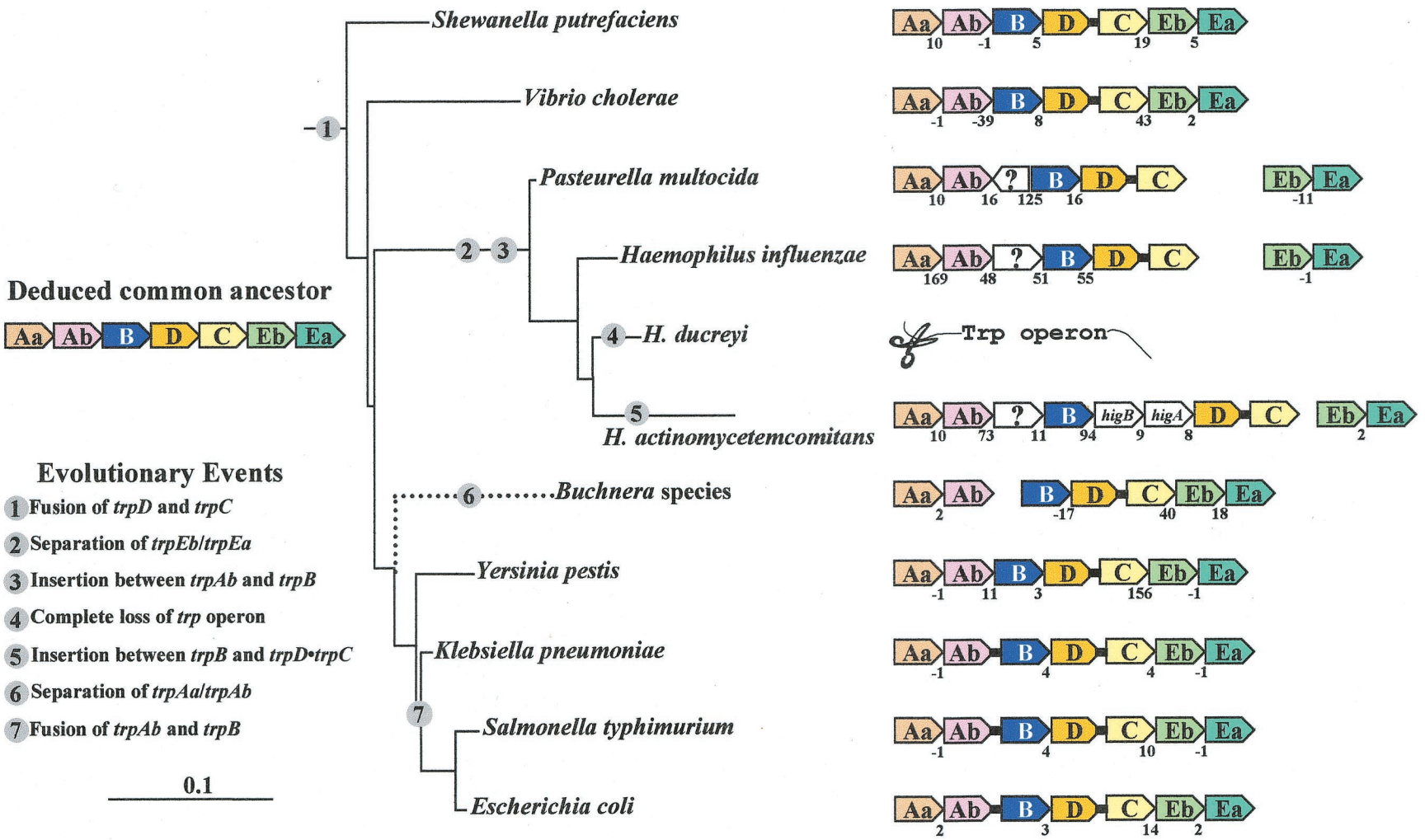


FIG. 8. Zoom-in from Fig. 6B showing Trp pathway gene organization in a range of Proteobacteria defined by the presence of the *trpD● trpC* fusion. Deduced phylogenetic events described on the left are identified by number on the 16S rRNA tree at the evolutionary times indicated. The actual position of *Buchnera* on the 16S rRNA tree (as shown in Fig. 2D and Fig. 4) is closest to *E. coli*. However, the long branch (Fig. 4) is consistent with the more likely order of branching depicted by the dotted line for *Buchnera* in this figure.

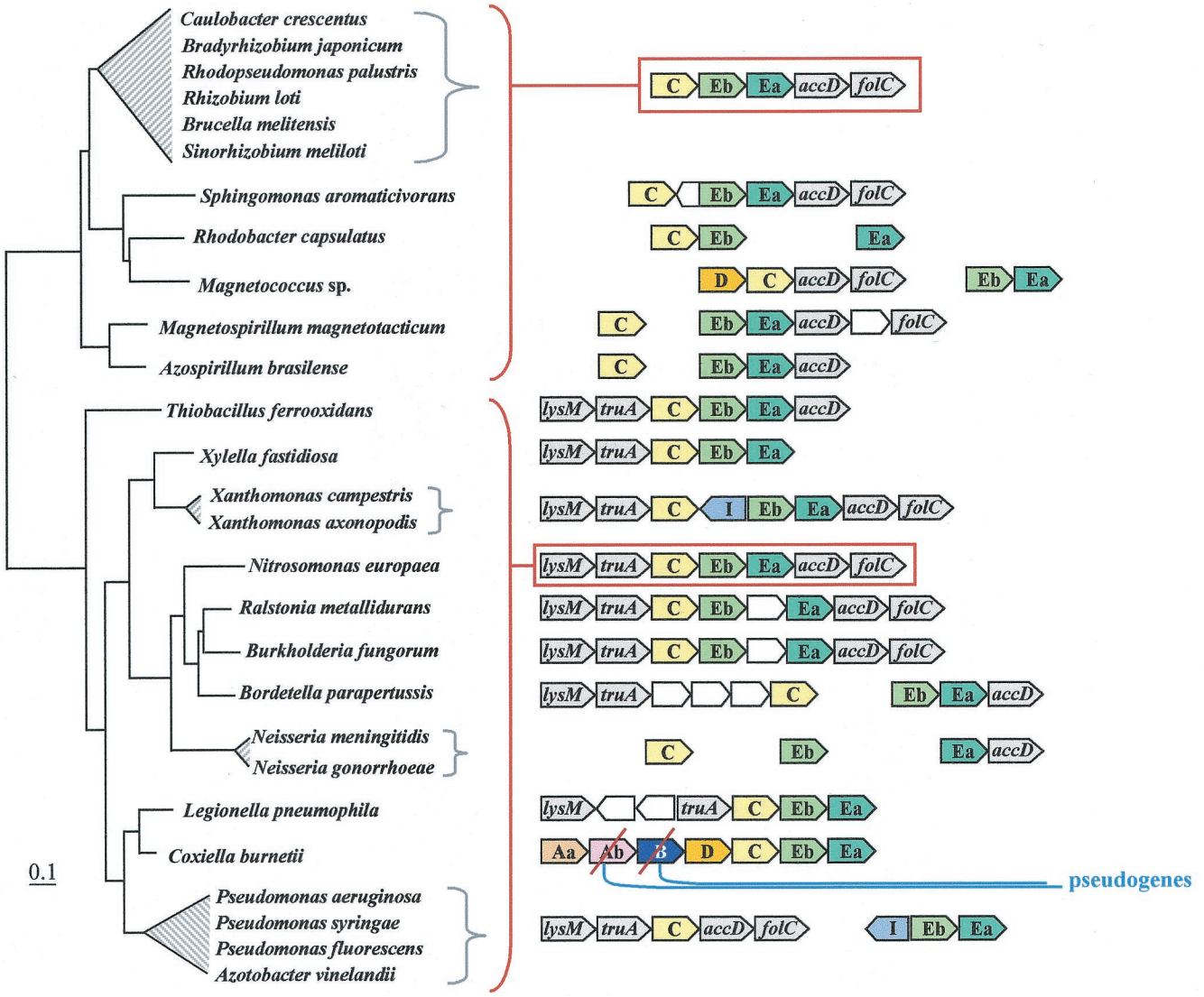


FIG. 9. Conserved genes flanking the *trpC/trpEb/trpEa* operon of organisms within the split-operon portion of the 16S rRNA tree. Organisms in the upper grouping are α-Proteobacteria; the cluster between *Thiobacillus* and *Neisseria* are β-Proteobacteria; and the bottom cluster is that fraction of the γ-Proteobacteria that diverged prior to the *trpD●trpC* fusion event. *lysM* and *truA*, conserved at the flanking gene position at the left throughout the β- and γ-Proteobacteria, are shaded grey, as are *accD* and *folC* (conserved in the flanking gene position at the right throughout the phylogenetic span portrayed in this figure). The deduced gene order of the common ancestor for each of the two major 16S rRNA clades is the same as shown for the two contemporary organisms *Rhodopseudomonas palustris* and *Nitorosomonas europaea*, as indicated by outlining in orange. Intervening genes, either hypothetical or known, are shown as open block arrows.

tial-pathway operon did exhibit flanking conserved genes. On the right in Fig. 9 are shown the positions of conserved genes that do flank the *trpC/Eb/Ea* operon. Genes encoding the β subunit of acetyl-coenzyme A carboxylase (*accD*) and folylpolyglutamate synthase/dihydrofolate synthase (*folC*) follow *trpEa* in most cases. Occasionally *folC* appears to have been translocated away from *trpEa/accD*, as exemplified in *Bordetella parapertussis* and *Neisseria meningitides* and *Neisseria gonorrhoeae*. For the lower group of organisms (from *Thiobacillus* through the *Pseudomonas/Azotobacter* cluster), the *trpC/Eb/Ea* operon is additionally flanked on the left by genes encoding fimbria V protein (*lysM*) and tRNA pseudouridine synthase A (*truA*). The top group of organisms shown in Fig. 9 exhibit the gene order *trpC/trpEb/trpEa/accD/folC* (boxed) that likely mirrors the ancestral gene order of the alpha-Proteobacteria, whereas it is reasonable to suggest that the gene order of *Nitrosomonas europeae* represents the ancestral gene order of the remaining organisms in the tree.

These conserved flanking genes provide information that can help guide fine-tuned evolutionary deductions. For example, the clade that includes *Pseudomonas aeruginosa*, *P. syringae*, *P. fluorescens*, and *Azotobacter vinelandii* possesses a *trpC* gene that has become separated from *trpEb/trpEa.* Was *trpC* or *trpEb/Ea* transposed away from the original *trpC/Eb/Ea* operon? The answer clearly is *trpEb/trpEa*, since *trpC* is flanked on the left by *lysM/truA* and on the right by *accD/folC*. Likewise, in the *Magnetococcus* sp., *trpC* is flanked on the right by *accD* and *folC*, and therefore the *trpEb/trpEa* operon must have been

translocated away from *trpC*. Also, *trpD* in *Magnetococcus* must have migrated from the *trpAa*/*Ab*/*D* operon to its anomalous contemporary position near *trpC*.

Both *Magnetospirillum* and *Azospirillum* resemble the *Magnetococcus* sp. in that *trpC* has separated from *trpEb*/*trpEa*. However, in contrast to *Magnetococcus* sp., in which *trpEb*/*trpEa* has been transposed, in both *Magnetospirillum* and *Azospirillum* it is *trpEb*/*trpEa* that is linked to *accD*, and therefore it is clearly *trpC* that has been transposed away.

In *Bordetella parapertussis trpC* has separated from *trpEb*/*trpEa* in such a way that *trpC* retains linkage with *lysM*/*truA* on the left and *trpEb*/*trpEa* retains linkage with *accD* on the right. This could be consistent with a very large insertion (49,000 bp) between *trpC* and *trpEb*, or more likely *trpEb*/*trpEa*/*aacD* were jointly transposed. In *Neisseria meningitides* and *Neisseria gonorrhoeae*, *trpC*, *trpEb*, and *trpEa* have all become separated from one another. In this case, *trpEa* has retained its linkage with *accD*.

EXPANDED METABOLIC CONTEXT

Biochemical pathways are complexly interlinked in a net-like fashion, as any wall chart reveals, and it is of interest to examine the organization of Trp pathway genes in the larger metabolic context of aromatic amino acid biosynthesis (90). While even this is a relatively elementary metabolic expansion, a comprehensive analysis of this is well beyond the scope of this paper. However, two examples are given below (one from the *Archaea* and one from the *Bacteria*) which illustrate that the evolution of Trp biosynthesis has not necessarily occurred in isolation from its immediate biochemical connections. It is important to appreciate that a fuller future understanding of Trp biosynthesis will ultimately extend to the larger scope of interlocking metabolic ties that exist. A fuller appreciation of the varied interlocking ties of Trp biosynthesis with its metabolic context should be quite relevant to understanding the selective pressure favoring or disfavoring LGT. In addition, one can expect that conservation of an existing operon system would be significantly strengthened by a full repertoire of integrated metabolic ties.

Pyrococcus and Its Archaeal Relatives

Convergent *trp* and giant *aro* operons of *Pyrococcus*. *Pyrococcus furiosus* possesses a truly remarkable array of linked genes for general aromatic biosynthesis (Fig. 10). These include not only genes encoding every common-pathway step, but also all genes specifically tied to phenylalanine, tyrosine, and Trp biosynthesis except for *pheA*. This even includes a gene encoding aromatic aminotransferase (denoted *aspC*). Incredibly, ties to the pentose phosphate pathway (the source of erythrose-4-phosphate) are reflected by the presence of linked genes for transketolase and for an ABC system of ribose transport. All of the genes encoding common-pathway steps are in the exact order of the reactions in the pathway. Shikimate kinase, encoded by $aroE_{II}$ (our designation), is an analogue class of kinase that is specific to *Archaea* (18). If one orients to tyrosine biosynthesis, all of the genes, beginning with transketolase (which generates erythrose-4-phosphate), are present in exact order through the final *tyrA* step. *aroQ* (chorismate mutase) and *aspC* (aromatic aminotransferase) are used for both phenylalanine and tyrosine biosynthesis. The *P. furiosus aspC* gene product has been shown experimentally to be utilized specifically for phenylalanine and tyrosine biosynthesis (84). The adjacent *trp* operon (see Fig. 4 for detail), with its many overlapping genes, is transcribed convergently from genes of the large *aro* operon.

Pyrococcus abyssi possesses exactly the same array of linked genes except that the *aroQ*/*aspC*/*tyrA* segment is absent from the genome (84). These genes are specific for tyrosine and phenylalanine biosynthesis. *P. furiosus* possesses a stand-alone copy of *pheA*, whereas *P. abyssi* lacks *pheA* altogether.

Dynamics of archaeal gene shuffling. It is suggestive that the gene orders within the largest archaeal linkage groups that represent either *Crenarchaeota* (*P. furiosus*) or the *Euryarchaeota* (*S. solfataricus*) show some similarities, and we speculate that the ancestral gene order might have resembled that of the *P. furiosus aro* operon. This speculation is influenced by the gene order ($aroA_{I\beta}$/*aroB*/*aroC*/*aroD*/*aroE*/*aroF*/*aroG*) of the closest neighbor of *S. solfataricus*, *Aeropyrum pernix*. The altered order of *aroC* and *aroG* in *S. solfataricus* may reflect derived transposition events. If *P. furiosus* does represent the ancestral order, deletion of *aspC* could have resulted in the *aroQ*•*tyrA* fusion in *S. solfataricus*, which must then have been inserted between the ancestral *trk*-β and $aroA_{I\beta}$. If so, the deleted *aspC* gene was then inserted into the *trp* operon of *S. sulfataricus* (see Fig. 5) to become the distal gene member of the operon. Whether the *trp* operon became associated with the convergently transcribed *aro* operon in the *Pyrococcus* lineage or whether the *trp* operon dissociated from the *aro* operon in the *Euryarchaeota* seem to be equally possible alternatives that await resolution with the advent of more closely spaced genome representation.

The two *Thermoplasma* species (*T. acidophilum* and *T. volcanium*) and the closely related *Ferroplasma acidarmanus* have two identical *aro* operons except that $aroA_{I\beta}$ is missing in *Ferroplasma* in comparison with the *aroQ*/*tyrA*/$aroA_{I\beta}$ operon of the *Thermoplasma* species (Fig. 10). It is quite intriguing that this $aroA_{I\beta}$ gene has been inserted into the *trp* operon of *F. acidarmanus* at the distal gene position (Fig. 5).

It is apparent that genes of both Trp biosynthesis (Fig. 5) and overall aromatic biosynthesis (Fig. 10) have been atypically dispersed in *Methanococcus jannaschii*. This is reminiscent of the tendencies toward gene dispersal seen in some but relatively few of the *Bacteria* (species of cyanobacteria, *Aquifex*, and *Chlorobium*). *Methanopyrus kandleri*, a relatively close relative of *M. jannaschii*, also has dispersed Trp pathway genes, with only *trpAa* and *trpAb* (20-bp gene overlap) being adjacent (data not shown).

Bacillus/*Staphylococcus* Clade

The entire clade shown in Fig. 11A is distinguished by having an *aroQ*•*aroA* fusion that is the basis for the novel allosteric pattern of sequential feedback inhibition of DAHP synthase by intermediary metabolites (43). The lack of this fusion in *Enterococcus*, *Streptococcus*, and *Lactococcus* is one of a number of reasons for our exclusion of these groupings from the *Bacillus*/*Staphylococcus* clade. The *aro* operons shown within shaded brackets in Fig. 11A exist within a general genomic region

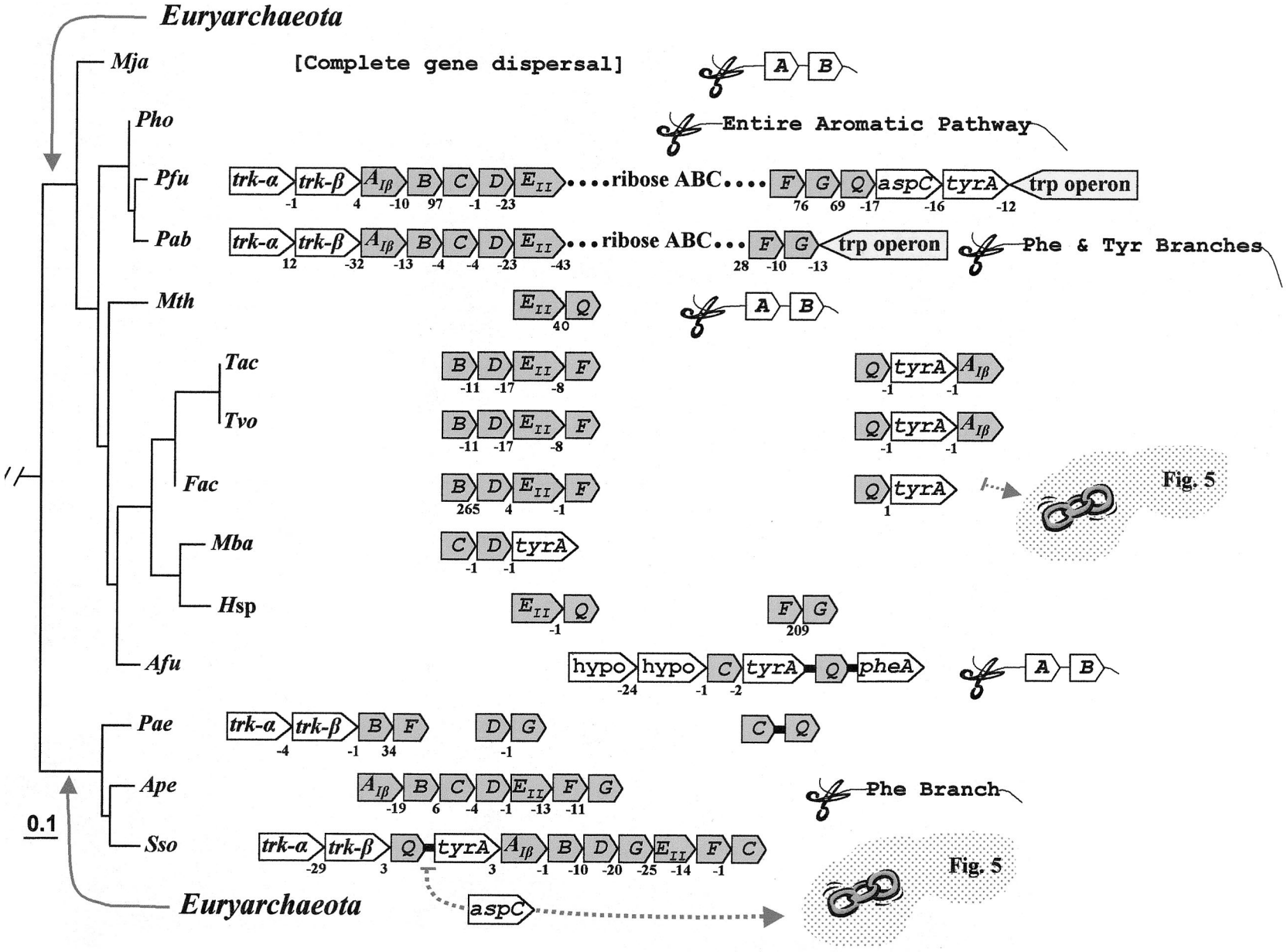


FIG. 10. Linkage relationship of genes within the larger context of aromatic amino acid biosynthesis in *Archaea*. The tree is the same as that shown in Fig. 5, where the full organism names corresponding to the acronyms used can be viewed. Common-pathway genes are shaded and designated by the gene letter, e.g., Q = *aroQ*. Hypothetical genes are denoted as hypo. Genes are labeled within block arrows that point in the direction of transcription. Copies of genes encoding transketolase are designated *trk*-α and *trk*-β. Short black bars connecting arrows indicate gene fusions. Deleted genes, pathway branches, and entire pathways are indicated with scissors.

between *gpsA* (encoding glycerol-3-phosphate dehydrogenase) and a conserved gene (*tpr*) encoding a TPR repeat-containing protein (shown in Fig. 11B). Throughout the entire clade, the gene order *gpsA*/*hbs*/*hepS*/*menH*/*hepT*/*ndk*/*aroG*/*aroB* is conserved.

***B. subtilis* subgroup.** *Bacillus subtilis*, a member of the lower of the two major subgroups shown on the left in Fig. 11A, possesses a well-studied (35) supraoperon in which the *trp* operon is nested within a larger transcriptional unit. The *B. subtilis trp* operon has lost *trpAb* but *pabAb* (originally called *trpX* [47]) has been shown to support the amidotransferase function for both anthranilate synthase and *p*-aminobenzoate (PABA) synthase, i.e., the TrpAa/PabAb complex functions as an anthranilate synthase and PabAa/PabAb/PabAc functions as a PABA synthase. The six-gene *B. subtilis trp* operon is very compact, with four points of translational coupling. It is flanked on the N-terminal side with three aromatic-pathway genes (*aroG*, *aroB*, and *aroH*) and on the C-terminal side with three additional aromatic-pathway genes ($hisH_b$, *tyrA*, and *aroF*).

$hisH_b$ (subscript denotes broad specificity) encodes a subgroup of imidazole acetyl aminotransferase that is widespread and functions as an aromatic aminotransferase (42). The other subgroup, $HisH_n$ (subscript denotes narrow specificity) functions in the pathway of histidine biosynthesis. Interestingly, the $hisH_b$/*tyrA*/*aroF* gene combination is part of another supraoperon (*serC*/$aroQ_p$●*pheA*/$hisH_b$/*tyrA*/*aroF*/*cmk*/*rpsA*) which has been characterized in *Pseudomonas stutzeri* and *P. aeruginosa* (90, 91). *aroH* is a relatively rare analogue class of chorismate mutase, thus far known to be present only in cyanobacteria and in a scattered distribution of gram-positive *Bacteria*, including, in addition to the lower group of *Bacillus* in Fig. 11A, *Desulfitobacterium hafniense*, *Carboxydothermus hydrogenoformans*, *Clostridium botulinum* (but not other *Clostridium* species), *Thermoanaerobacter tengcongensis*, *Streptomyces coelicolor*, *Thermomonospora fusca*, and *Heliobacillus mobilis*. The gene organizations of the *Bacillus halodurans* and *Bacillus stearothermophilus* supraoperons are essentially identical to that of *B. subtilis*. However, note that in *B. stearothermophilus* a conspicuous expansion of intergenic space between *trpC* and *trpEb* and between *trpEb* and *trpEa* is evident (Fig. 11A). We can be fairly sure, because of parsimony principles applied to the comparative data, that this intergenic expansion is a derived evolutionary state rather than an ancestral one.

Upstream of the *B. subtilis* supraoperon is the *mtrA*/*mtrB* operon, encoding GTP cyclohydrolase I and the TRAP regulatory protein, respectively (Fig. 11B). *mtrB* is uniquely present within the lower subgroup. *B. stearothermophilus* has conserved the general region shown in Fig. 11B between *gpsA* and the supraoperon, but *tpr* and its flanking region to the right have been transposed away. *B. halodurans* exhibits a number of unique insertions in the conserved region shown in Fig. 11B.

***Listeria* subgroup.** In spite of the current generic naming, *Bacillus anthracis* is closer on the 16S rRNA tree to species of *Staphylococcus* and *Listeria* (upper group in Fig. 11A) than to the other *Bacillus* species of the lower group. Members of this upper group all possess a complete seven-gene *trp* operon, including *trpAb*, which is absent in the lower *Bacillus* grouping of Fig. 11A. The *Staphylococcus*/*B. anthracis* group lacks the tryptophan RNA-binding attenuator protein (TRAP) encoded by *mtrB* (29), which is present throughout the lower group. The *Staphylococcus*/*B. anthracis* group also differs from the lower group and *Listeria* in the absence of *aroH*.

The *aroH* gene may be in a general process of displacement by *aroQ*, which is by far the most ubiquitous gene encoding chorismate mutase (13). Indeed, even within the lower group, one widely used strain of *B. subtilis* (strain 168) has lost *aroH* and relies exclusively on *aroQ* (48). The strain 168 genome, which has been sequenced and reported to possess *aroH* (as shown in Fig. 11A), is actually a hybrid prototrophic transformant with *B. subtilis* strain 23, the donor of *aroH* and linked *trp* genes (48). In *Staphylococcus* species of the upper group of Fig. 11A, the presumptive ancestral $hisH_b$/*tyrA*/*aroF* linkage group has been disrupted, and *aroF* is now linked to *aroG*/*aroB*, whereas *tyrA* is now linked (divergently) with an intact *trp* operon. In contrast, *B. anthracis* retains the $hisH_b$/*tyrA*/*aroF* linkage, but this has been expanded by addition of a gene duplicate of *aroG* at the 3′ end. In addition, the putative ancestral *aroG*/*aroB* has acquired a duplicate of $hisH_b$ at the 5′ end.

Note that we can distinguish which paralogues of *aroG* and $hisH_b$ in *B. anthracis* have remained in flanking gene context and which have been transposed away, i.e., the bracketed *aroG*/*aroB*/$hisH_b$ operon of Fig. 11A exists within the context shown in Fig. 11B. If *aroH* was present in the common ancestor of the clade, as speculated at the bottom of Fig. 11A, then it was lost in the common ancestor of the upper group. Otherwise, it arrived in the lower group either as a newly evolved innovation or by LGT. The first alternative may be more likely, considering that some fairly close relatives outside of the clade shown (e.g., *Clostridium botulinum* and *Thermoanaerobacter tengcongensis*) possess *aroH*.

Interconnectivity of the *trp*, *aro*, *pab*, and *his* operons. Figure 11 illustrates that organisms like *Listeria* and *Oceanobacillus* possess six-gene *aro* and seven-gene *trp* operons that are located in widely spaced parts of their genomes. They also have *pab* operons and *his* operons (not shown) that altogether constitute four separately spaced and seemingly unrelated operons. This presumably represents the straightforward ancestral state of the clade. In the *B. subtilis* clade, however, these separate operon systems have become integrated via the following events. (i) The *trp* operon was inserted into the *aro* operon to produce the well-studied supraoperon. (ii) $hisH_n$, a substrate-specific imidazole acetol phosphate aminotransferase, was deleted from the *his* operon, making the histidine pathway dependent upon $HisH_b$, a broad-specificity imidazole acetol phosphate aminotransferase encoded by the *aro* portion of the supraoperon. (iii) *trpAb* was deleted from the *trp* operon, leaving the Trp pathway dependent upon the dual-function PabAb encoded from the *pab* operon. A metabolic basis for integration of the *aro*, *trp*, and *pab* operons is readily apparent in that the component genes are all part of the divergently branched pathway of aromatic biosynthesis. A metabolic relationship between the aromatic and histidine pathways is not as straightforward. However, both have a precursor relationship with pentose phosphate metabolism, both utilize a glutamine amidotransferase reaction, and both utilize PRPP as a key early substrate.

Evolutionary information derived from flanking-gene context. Figure 11B shows a conserved region between *gpsA* and *tpr* that is the location of the six-gene *aro* operon in *Listeria* and *Oceanobacillus*. Upstream between the highly conserved *hbs* and *hepS* are *mtrA* and *mtrB* (if present). The shaded brackets

in Fig. 11A indicate the genes that are present within the flanking gene context detailed in Fig. 11B. In the major upper group, the *trp* operon has no consistent pattern of flanking genes. In *B. subtilis* and *B. halodurans*, the supraoperon genes are ordered within the region shown on the bottom line of Fig. 11B. In *B. anthracis*, an *aroG*/*aroB*/*hisH*$_b$ segment of the original six-gene *aro* operon has remained in the original context of flanking genes. Paralogues of *aroG* and *hisH*$_b$, now associated with *tyrA* and *aroF*, have migrated to a new genomic position. In *Staphylococcus* the remnant of the original *aro* operon, *aroG*/*aroB*/*aroF*, has remained in the original context of flanking genes; *aroH* has been lost from the genome; and *hisH*$_b$ and *tyrA* have separately been moved elsewhere. In the case of *tyrA*, it has now been divergently positioned directly upstream of the *trp* operon.

Thus, both this analysis and the analysis represented by the data shown in Fig. 9 illustrate how flanking-gene context in relatively close sister lineages can help sort derived evolutionary events from ancestral ones.

Deducing the likely common ancestor of the clade. Thus, the major upper and lower groups of Fig. 11A differ in the gene organization of the *trp* operon (presence or absence of *trpAb*), in the regulation of the operon (presence or absence of *mtrB*), and in the particular context of association with other aromatic-pathway genes (Fig. 11B). The most conserved gene order arrangements overall, in addition to the *trp* operon, are *aroG*/*aroB* and *hisH*$_b$/*tyrA*/*aroF*. One can be fairly certain that the common ancestor possessed the complete *trpAa*/*Ab*/*B*/*D*/*C*/*Eb*/*Ea*, *aroG*/*aroB*, and *hisH*$_b$/*tyrA*/*aroF* gene orders. This is because the linkage of *aroG*/*aroB* persists throughout the organisms shown in Fig. 11A and because the *hisH*$_b$/*tyrA*/*aroF* linkage is well conserved, even at a deeper level, in the *Bacteria*. Deduction of a convincing common ancestor will require the genome sequences of additional organisms that will present a more finely spaced phylogenetic progression. A case in point that illustrates the process was our recent consideration of the new genome sequence for *Thermoanaerobacter tengcongensis* in this connection. When the Blast similarities of proteins from *T. tengcongensis* were scored against the overall genomic database (8), the highest score was for *B. halodurans*. Had this reflected membership of *T. tengcongensis* in the Fig. 11A clade, as we anticipated, it might have assisted deduction of evolutionary events in the clade. However, *T. tengcongensis* does not have the clade-conserved *aroQ*●*aroA* fusion, and its position on the 16S rRNA tree also places it outside the clade.

Given the tentative deduced ancestral linkages shown at the bottom of Fig. 11A, evolution of the supraoperon of the lower group must have involved loss of *trpAb* and the connection of *aroG*/*aroB*/*aroH* at the 5′ end of the operon, as well as joining of *hisH*$_b$/*tyrA*/*aroF* at the 3′ end of the operon. If the common ancestor possibly possessed *aroG*/*aroB*/*aroH*/*hisH*$_b$/*tyrA*/*aroF* as a single linkage group (as seems probable in view of the presence of this six-gene *aro* cluster in *Listeria* and *Oceanobacillus*), a single event of insertion of the *trp* operon between *aroH* and *hisH*$_b$ would account for the contemporary supraoperon. We propose that the gene organization of the common ancestor of the clade shown in Fig. 11A was very similar to that of the modern *Listeria monocytogenes.*

OVERVIEW PERSPECTIVES

Lineage-Specific Evolutionary Trends

There may be lineage-specific forces at work that have favored processes of gene dispersal, operon fragmentation, and gene insertion for reasons that are currently unappreciated. When considering in a comparative context the intact and highly compact Trp and His operons of *E. coli*, we noticed that various *trp* operon features (which are comprehensively documented in this paper) seem to exhibit parallel differences with respect to histidine operon features. Thus, it seems more than coincidence (i) that both the Trp pathway genes and the histidine pathway genes are dispersed in *Aquifex* and in the unicellular cyanobacteria, (ii) *Campylobacter jejuni* has an intact *his* operon except for the dissociation of *hisC*, reminiscent of its otherwise intact *trp* operon which exhibits dissociation of only *trpD* (Fig. 6A), (iii) *P. aeruginosa* exhibits fragmentation into four partial-pathway operons of histidine biosynthesis (*hisGDC*, *hisBHAF*, and *hisIE*) reminiscent of its fragmentation into partial-pathway operons for Trp biosynthesis (Fig. 6B), and (iv) *Lactococcus lactis* exhibits seemingly extraneous multiple gene insertions in its complete *his* operon (2), similar to insertions observed in its complete *trp* operon.

The comparative analysis of the histidine operon is well beyond the scope of this paper, but dynamics of gene scrambling similar to those seen with the *trp* operon are evident, e.g., the *E. coli* gene order *hisG*/*D*/*C*/*B*$_d$●*B*$_{px}$/*H*/*A*/*F*/*I*●*E* compared to the *Sulfolobus solfataricus* gene order *hisC*/*G*/*A*/*B*$_d$/*F*/*D*/*E*/*H*/*I*/*B*$_{px}$. A preliminary assessment indicates that the histidine pathway gene organization exhibits some intriguing parallels to Trp pathway gene organization. Different events of gene scrambling, gene dispersal, gene fusion, intergenic expansion, and operon fragmentation exist in both *Bacteria* and *Archaea*. Similar to what seems to be the case for the Trp pathway gene organization, gene scrambling also seems to be more frequent for histidine pathway gene organization in the *Archaea* than in the *Bacteria*.

Individual Divergences Unmasked in the Larger Genomic Context

Figure 12 portrays the relationship of a few selected organisms with respect to the overall deduced evolutionary histories of the *trp* operon. The three major *trp* operon gene organizations are displayed within color-coded ovals that correspond to the highlighting of specific organisms in Fig. 2. Subsequent evolutionary events deduced for selected organisms emerging from each group are shown. The intent is to illustrate how detailed case-by-case analyses can elucidate evolutionary histories that would not be at all apparent otherwise. Thus, the *trpAa*/*Ab*/*B*/*D*/*C*/*Eb*/*Ea* operon of *Coxiella burnetii* (lower left) at first inspection appears to have experienced no evolutionary change because it is identical to the deduced ancestral operon. However, our analysis indicates the intervention of two evolutionary events, one producing the two "split-pathway operons" present in most Proteobacteria and the second rejoining the two previously separated operons.

As a second example, a comparison of the *E. coli trp* operon with the ancestral *trp* operon reveals only two differences in structural gene organization, fusion of *trpD* with *trpC* and fu-

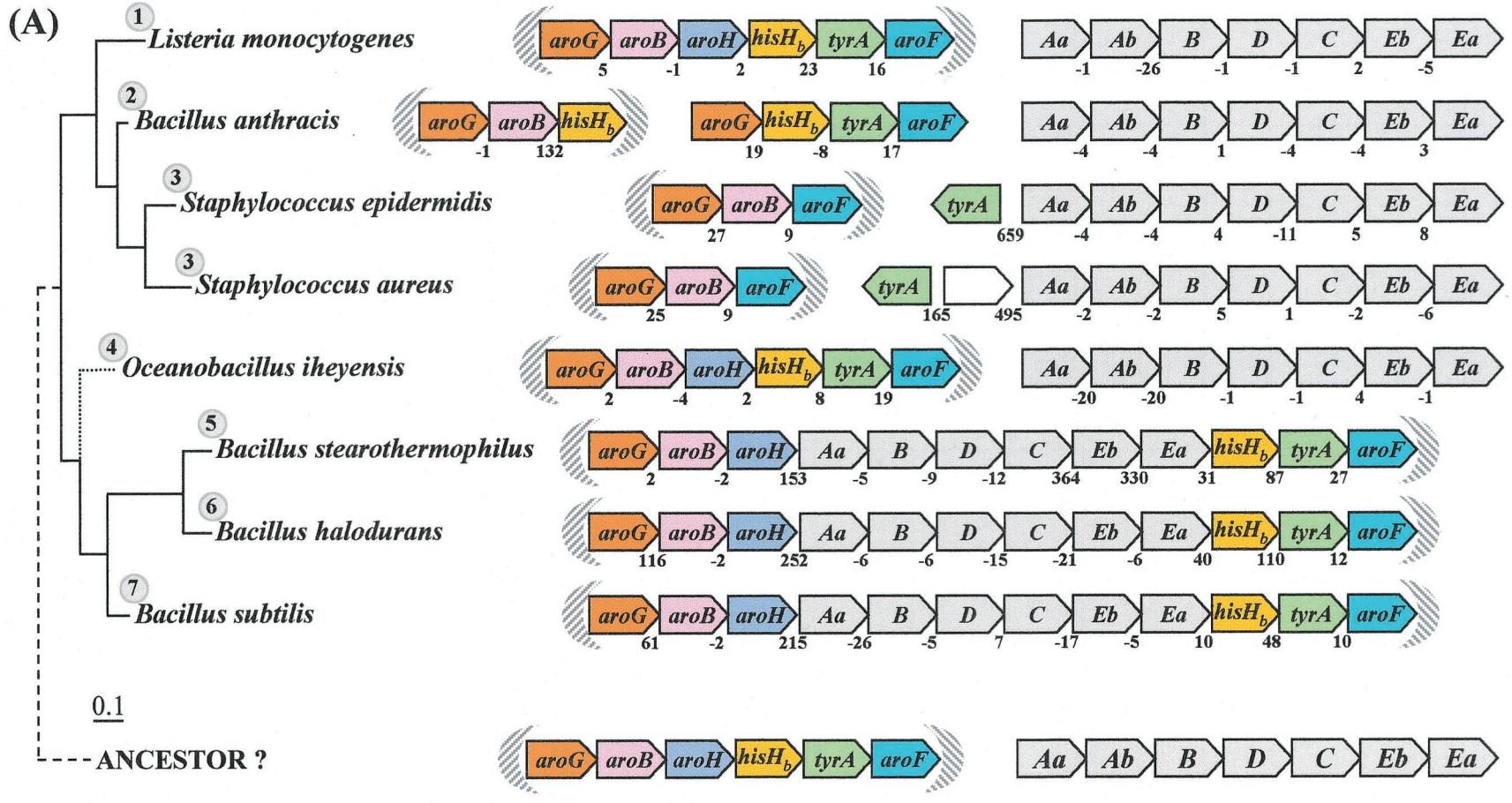

(A)
1 Listeria monocytogenes
2 Bacillus anthracis
3 Staphylococcus epidermidis
3 Staphylococcus aureus
4 Oceanobacillus iheyensis
5 Bacillus stearothermophilus
6 Bacillus halodurans
7 Bacillus subtilis
0.1
ANCESTOR ?
aroG aroB aroH hisH$_b$ tyrA aroF
Aa Ab B D C Eb Ea
hisH$_b$ tyrA
aroF
(B)
1 Lmo
2 Ban
3 Sau/Sep
4 Oih
5 Bst
6 Bha
7 Bsu
gpsA
spoIVA
hbs
mtrA
mtrB
hepS
menH
qpt
acd
hypo
hepT
ndk
cheR
Trp operon
tpr

FIG. 11. Zoom-in from Fig. 6A showing a conserved gram-positive region containing the six-gene *aro* operon (or remnants of it) and the *trp/aro* supraoperon of the *B. subtilis/B. halodurans/B. stearothermophilus* subgroup. (A) The *aro* and *trp* operons are mapped on a 16S rRNA tree at the far left. (The exact branching order of *Oceanobacillus iheyensis* has not been determined.) The *Enterococcus/Streptococcus/Lactococcus* grouping branches off between *Listeria* and the *B. anthracis* subgroup on a 16S rRNA tree (not shown, but see Fig. 2B and Fig. 7), but we believe from a variety of observations that it belongs just outside of the lineage shown in this figure. Shaded bracketed regions around *aro* operons and *trp/aro* supraoperons can be related to the presence of a context of conserved, flanking genes, as shown in part B. The separate *aro* and *trp* operons of a putative common ancestor are shown at the bottom of A. *aro* genes in B are color coded to match the genes shown in A. The conserved region to the left of *aro* operon genes includes eight genes (*gps*, *hbs*, *hepS*, *menH*, *hepT*, *ndk*, *aroG*, and *aroB*) that are conserved in every organism shown (heavy black overbars). Gene abbreviations: *gpsA*, glycerol 3-phosphate dehydrogenase; *spoIVA*, sporulation protein IVA; *hbs*, nonspecific DNA-binding protein; *mtrA*, GTP cyclohydrolase I; *mtrB*, TRAP; *hepS*, heptaprenyldiphosphate synthase (component I); *menH*, heptaprenyl naphthoquinone methyltransferase; *qpt*, quinone polyprenyltransferase; *acd*, aromatic acid decarboxylase; *hypo*, hypothetical gene; *hepT* heptaprenyldiphosphate synthase (component II); *ndk*, nucleoside diphosphate kinase; *cheR*, chemotaxis protein methyltransferase, *tpr*, tetratricopeptide repeat-containing protein (COG0457).

sion of *trpAb* with *trpB*. However, we have shown that the ancestral operon must have split into the two halves shown in Fig. 12 prior to the *trpD●trpC* fusion. In this connection, the *trp* operon of *Thermotoga maritima* differs from the ancestral operon only in having the *trpAb●trpB* fusion. With limited information, one might have predicted that the *T. maritima* operon was directly intermediate between the ancestral state and the *E. coli* state, i.e., the ancestral state, followed by the *Thermotoga* state (*trpAb●trpB* fusion), followed by the *E. coli* state (*trpAb●trpC* fusion). However, we have found that the two *trpAb●trpB* fusions occurred independently. The contemporary operons of *T. maritima* and *E. coli* do not show any common steps of operon change, and a much richer evolutionary history exists than would be evident from superficial inspection.

The well-studied *trp* operons of *Pseudomonas aeruginosa* and *Bacillus subtilis* are illustrated in Fig. 12 as examples of operons from organisms that are not representative of the deeper phylogenetic node. Since the time of the landmark splitting of the ancestral operon, a history of additional fragmentations in *P. aeruginosa* has left *trpAa* isolated from *trpAb*/*B*/*D* and *trpC* isolated from *trpEb*/*Ea*. Likewise, the well-studied *trp* operon of *Bacillus subtilis* is not representative of the broader *Listeria*/*Bacillus*/*Staphylococcus* node. In relatively recent events, *trpAb* has been discarded, and the remaining *trp* operon appears to have been inserted into an *aro* (*aroG*/*aroB*/*aroH*/*hisH*$_b$/*tyrA*/*aro*/*F*) operon. (see Fig. 11 and the attending discussion in this text). Since the dual use of *pabAb* in the lower group for both anthranilate and PABA synthesis is isolated to this lineage, the seven-gene *trp* operon of *Bacillus anthracis* and *Staphylococcus* species is more representative of the node of Fig. 11 organisms than is the six-gene *B. subtilis* operon.

Analysis of the Ancestral State at Phylogenetic Nodes

Our study illustrates how one can avoid errors due to LGT and ancient paralogy and identify the most likely common ancestor that represents a phylogenetic node. If nodes at the bottom of the tree are sufficiently well represented to deduce the state of the *trp* operon at those nodes, one can deduce the likely common ancestor at progressively more ancient nodes, working backwards in evolutionary time up the tree. This is illustrated by zoom-in figures in relationship to the mapping of Trp pathway genes on the 16S rRNA tree for *Bacteria* in Fig. 6. Thus, to give some examples of cases where evolutionary differences in closely related members of a clade can be distinguished as ancestral states or derived states, we have seen (i) that the *trp* operon of *Listeria monocytogenes* but not *B. subtilis* is representative of the node position of the common ancestor for the *Listeria*/*Lactococcus*/*Staphylococcus*/*Bacillus* clade (Fig. 7 and 11), (ii) that the two partial-pathway operons of *Thiobacillus ferooxidans* and *Methylococcus capsulatus* but not the *trp* gene arrangements of *Pseudomonas aeruginosa* or *Neisseria meningitidis* are representative of the ancestral state at the node representing those Proteobacteria (Fig. 6B) that diverged after the major event of operon splitting (Fig. 12), (iii) that *Shewanella putrefaciens* is more representative of the phylogenetic node for enteric bacteria than *Haemophilus influenzae* (which is probably undergoing an early phase of reductive evolution) or *E. coli* (which has experienced a recent additional gene fusion); and (iv) that *Campylobacter jejuni* is more rep-

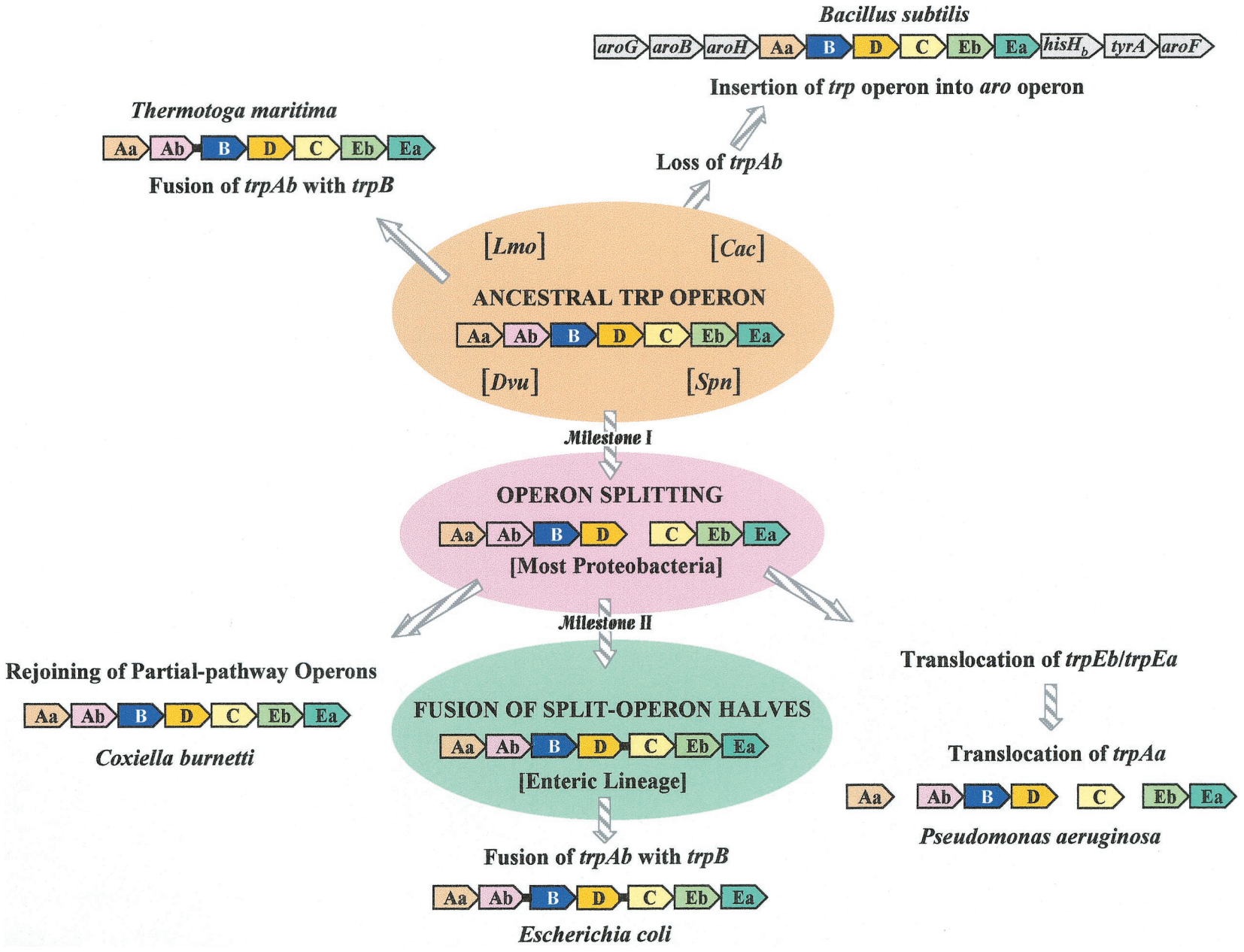


FIG. 12. Schematic of the major evolutionary events (milestone I and milestone II) following the ancient establishment of a *trp* operon in the domain *Bacteria*. The ancestral *trp* operon has been retained by organisms such as *Listeria monocytogenes* (Lmo), *Clostridium acetobutylicum* (Cac), *Streptococcus pneumoniae* (Spn), and *Desulfovibrio vulgaris* (Dvu). The emergence of selected contemporary organisms is shown. The three stages highlighted with an orange oval, a magenta oval, and a green oval correspond to the color coding used in Fig. 2 to designate the particular contemporary organisms that have retained the exact gene organization illustrated within one of the three ovals.

resentative of its common node with *Helicobacter pylori* because the native *trp* operon of *H. pylori* was displaced by an alien *trp* operon via LGT.

The clade of actinomycete *Bacteria* shown in Fig. 3 offers a particularly apt example of how genes representing the ancestral state of Trp biosynthesis can be sorted out from genes originating by LGT or ancient paralogy. The *Thermomonospora*, *Streptomyces*, *Corynebacterium*, and *Mycobacterium* genera each exhibit substantial differences from one another. *Mycobacterium* lacks paralogue copies of *trp* genes, and therefore its *trpAa*/*D*/*Eb*/*Ea* operon plus dispersed copies of *trpAb*, *trpB* and the missing *trpC* that are present can reasonably be assumed by default to specify the primary pathway of Trp biosynthesis. The situation is the same in *Thermomonospora* except that *trpAa* and *trpAb* are fused. *Streptomyces* possesses several *trp* operons, but the primary *trpAa*/*D*/*Eb*/*Ea* biosynthetic operon can be identified by phylogenetic analysis. Thus, proteins encoded by each of the *trpAa*/*D*/*Eb*/*Ea* operons as well as the free-standing copies of *trpAb* and *trpB* in the organisms shown in Fig. 3 all cluster together on phylogenetic trees to the exclusion of other paralogues present in the *Streptomyces* genome.

The *trpAa*/*Ab*/*B*/*D*/*aroA*$_{II}$ operon is known to have a specialized role in antibiotic production that is unique to *Streptomyces*. The free-standing TrpD of *S. coelicolor* specifically clusters in the phylogenetic tree with TrpD proteins encoded by the *trpAa*/*D*/*Eb*/*Ea* operons in the rest of the clade. It is a *trpD* remnant, since all other genes have been otherwise replaced by a whole-pathway operon via LGT. Thus, with all of this information, we can reasonably predict that the common ancestor at the node position for actinomycete bacteria (as depicted in Fig. 6) possessed a *trpAa*/*D*/*Eb*/*Ea* operon, with the remaining *trp* genes dispersed.

Intellectual Dilemma Addressed

Does *trp* gene reorganization necessarily imply functional deterioration? A central dilemma that merits consideration was posed in the Introduction. *trp* operons of model organisms such as *E. coli* and *B. subtilis* are elegantly geared for the efficient regulation of what is the most biochemically expensive of the 20 amino acids. As such, one might think that once evolved, forces of selection would enforce stability of the first order. Therefore, the variety and frequency of *trp* operon rearrangements, which have involved events of gene shuffling, gene fusion, operon splitting, total gene dispersal, and insertion of seemingly unrelated genes, is a dilemma that underlies this study. Since the overwhelming majority of modern prokaryote lineages maintain whole-pathway or at least partial-pathway *trp* gene organizations, the operon surely must generally constitute a selective advantage. To what extent do all of these changes imply operon disruption as opposed to fine-tuned improvement (or neutrality) of the operon system? Among all of the types of change, only total gene dispersal, as occurred, for example, in unicellular cyanobacteria, clearly constitutes an event of operon disruption. The cleavage of whole-pathway operons to yield two or more partial-pathway operons would seem disadvantageous, but this may reflect an evolutionary strategy of which we are presently unaware. Certainly the multiple control mechanisms used to control three different *trp* transcriptional units in *P. aeruginosa* hint at this.

Events of gene insertion and gene shuffling are not necessarily events of gene disruption. The reshuffled deck of *trp* genes in an operon such as that of *Desulfitobacterium hafniense* (Fig. 6A) seems curious, indeed, but there is no reason to believe that this compact operon is any less efficient for the shuffling. Perhaps the shuffling reflects nature's continuing experimentation to test for different orders of translationally coupled genes that produce different protein-protein interactions. When previously compact operons are altered by expansion of intergenic spacing, perhaps this is a necessary evolutionary step for successful gene fusion. Sufficient intergenic space would seem to be necessary for evolution of a linker region that does not intrude on the catalytic domains being fused.

Are there any clear examples of efficient operons systems that have been disrupted? We do not know the extent to which the high efficiency of regulation that is fully documented in only a few organisms such as *E. coli* and *B. subtilis* is typical of other *trp* operons. The regulatory features of *E. coli* and *B. subtilis* are distributed within rather narrow clades, and it may be that these exemplify relatively recent advanced operon systems that will in fact strongly resist future disruptive events in all of the free-living descendants. It would be most informative to know the details of regulation in a well-spaced phylogenetic progression of other modern whole-pathway operons (such as the operons carried by the orange-highlighted organisms in Fig. 2). For example, a two-component response regulator gene is positioned only 17 bp upstream of *trpAa* in *Thermotoga maritima*. Might this reflect the presence of a completely different mode of control?

It is possible that many *trp* operons in nature are relatively primitive and only have the advantages conferred by a common promoter and (in the case of overlapping genes) either translational coupling or protection from mRNA degradation. It seems quite probable that many free-living organisms have no use for the huge range of *trp* gene expression that is typical of a feast-and-famine organism such as *E. coli*. For example, cyanobacteria probably make most or all Trp endogenously and thus may require regulation over a minimal range. One could envision that simple feedback inhibition of anthranilate synthase might constitute the main regulation in operation. This is consistent with the results of two studies of cyanobacteria in which exogenous Trp transport was two orders of magnitude less than in *B. subtilis* (32), anthranilate synthase is 100% inhibited at 10 μM Trp (36), and the range of enzyme expression varies only two- to threefold except for a 20-fold range in the case of tryptophan synthase (36).

There are distinct examples where operon disruption has followed acquisition of a finely tuned *trp* operon system, e.g., dissociation of *trpEb*/*trpEa* in *Pasteurella multocida* in the enteric lineage (see Fig. 8). However, these are special cases in organisms that have become pathogens or intracellular symbionts. There is ample evidence that evolved interorganismal relationships can produce completely new selective conditions that no longer require an efficient operon. In the extreme case, many pathogenic organisms undergo reductive evolution and abandon the pathway altogether because the host provides Trp. Since eukaryote hosts (such as humans) are relatively recent, such processes are likely to be in an ongoing state. In these cases, events of gene insertion and gene dissociation may not be selectively disadvantageous. Indeed, they may be steps

in the selected process of genome reduction. In this connection it might be instructive to consider the recent disruptive events that have occurred in the pathogenic *Corynebacterium diphtheriae* but not in the free-living sister species *Corynebacterium glutamicum* since the LGT-mediated acquisition of the *trp* operon in their common ancestor (see Fig. 3 and attending discussion).

In a completely different context of interorganismal relationship, *Buchnera aphidicola* is an endosymbiont that produces Trp for the host. In this case, one can pinpoint a fairly recent time of selection against efficient regulation of Trp biosynthesis. Here the endosymbiont cells have been challenged to overproduce Trp for export to the host. This is primarily accomplished by translocation of *trpAa*/*trpAb* to a plasmid, with the result of giving a 16-fold amplification of the rate-limiting first step of Trp biosynthesis (50). It is very important to keep in mind that genomic sequencing has been heavily biased in favor of organisms that directly impact humans, and genomic representation of free-living organisms is still relatively weak.

The answer to the question raised in the heading is then yes and no. Pathogens (especially obligate pathogens) are in the process of abandoning the *trp* operon altogether. Endosymbionts, such as *Buchnera*, may abandon the regulation altogether in order to engineer themselves to saturate the needs of the host. However, there is thus far no evidence that a free-living organism equipped with a highly evolved and efficiently regulated *trp* operon experiences instability with respect to that operon.

Elaborate regulation seems to be fairly recent. Primitive *trp* operons may have been regulated by relatively simple schemes. Consistent with this is that all elaborate control systems for *trp* operons are restricted to marrow clades. The advanced *trp* operon of *E. coli* differs from that of the putative common ancestor of *Bacteria* in having two pairs of structural gene fusions (Fig. 4), the *trpR* repressor, and a leader peptide (*trpL*) for attenuation. The distribution of *trpR* is limited to the enteric lineage except for *Coxiella burnetii*, *Xylella fastidiosa*, and some species of *Chlamydia* (89). Regulation by attenuation mechanisms seems to be distinctly more widespread than repression control by *trpR* (7, 53, 75). However, particular attenuation mechanisms can be distinctly different. Thus, the mechanism in *E. coli* that relies on the *trpL* leader peptide (95) is quite distinct from the *Bacillus subtilis* mechanism that utilizes a Trp-activated RNA-binding protein (TRAP) (29) as well as an anti-TRAP protein whose synthesis is induced by uncharged $tRNA^{Trp}$ (80).

Does the enteric clade (see Fig. 8), with its multiple mechanisms of control, perhaps possess a relatively superior *trp* operon that would resist future events of operon disruption? It may very well be that the enteric lineage (as represented in Fig. 8) currently has a very highly conserved operon system in its free-living members. Exceptions in pathogenic organisms that are undergoing reductive evolution are easily understood (e.g., *Haemophilus* species), as are exceptions in intracellular symbionts such as *Buchnera*.

The *L. lactis* tRNA-directed transcription termination mechanism might prove to be the most broadly distributed mechanism, since various gram-positive organisms utilize this mechanism for a number of different amino acid biosynthetic pathways (34). The loss of *trpAb* from the *trp* operon of the *B. subtilis* clade and reliance upon the broad-specificity homologue in the folate pathway for dual function in anthranilate and 4-aminobenzoate synthesis may have favored an even more advanced regulatory system that integrates folate and Trp biosynthesis. In accord with this, TRAP also regulates the transcript levels in the *B. subtilis* folate operon (20).

Regulation of Trp biosynthesis in organisms lacking the whole-pathway operon may be relatively undeveloped aside from the widespread sensitivity of anthranilate synthase to feedback inhibition by Trp. Several partial-pathway operons are known to possess only a degree of regulation. Thus, in *Rhizobium meliloti*, the *trpAa●trpAb* operon is regulated by transcription attenuation but not the *trpBD* operon or the *trpCEbEa* operon (7). However, such a generalization may not be justified in consideration of *P. aeruginosa* and its close relatives *P. putida*, *P. fluorescens*, *P. syringae*, and *Azotobacter vinelandii*, in which transcription of the *trpEbEa* operon is activated by *trpI* (6, 14) and the free-standing *trpAa* and the *trpAbBD* operon are regulated by attenuation (67).

Given the variety of *trp* operon regulatory mechanisms that are known to have evolved and others undoubtedly yet to be discovered (30), one might think that selection for the most efficient operons would have proceeded rapidly via LGT. This may be an oversimplification in that different levels of efficiency may be selected for different lifestyles. Feast-and-famine organisms such as *E. coli* may be most suited to relatively large ranges of control modulation. In any event, only the LGT relationship of whole-operon transfer between *Helicobacter pylori*, coryneform bacteria, and enteric bacteria is evident at present. An obvious roadblock to LGT of at least some complexly regulated operons is the presence of regulatory genes at unlinked loci with respect to the operonic structural genes. It may very well be (see following section) that what is efficient in the metabolic context of one lineage is not so efficient in the metabolic context of another lineage (40).

Regulation extending beyond the Trp pathway. From the vantage point of operon stability, we think that it is very important to consider how deeply some modern *trp* operon systems have become integrated into a broader metabolic network. The first example is *trpR* in *E. coli*. Not only the *trp* operon but also four additional transcription units belong to the *trpR* regulon (68). Other members of the regulon include the *trpR* gene itself (which is therefore autoregulated), *mtr* (encodes a Trp-specific transporter), *aroL* (encodes shikimate kinase II), and *aroH* (a paralogue of the DAHP synthase $AroA_I\alpha$ homology group that is also feedback inhibited by Trp). *aroL* is also a member of the *tyrR* regulon. Thus, fine-tuned regulation by *trpR* is not only focused upon the specific Trp branch, but also influences the broader aromatic pathway, which generates precursor molecules. There is a certain integrant relationship in which the presence of *trpR* correlates with multiple, differentially regulated isoenzymes of DAHP synthase. It may be relevant here that there is a correlation between disruption of the whole-pathway *trp* operon in *Haemophilus influenzae* and the loss of genes encoding two of the three differentially regulated isoenzymes of DAHP synthase that are typically present in enterics.

The second example is that of *mtrB* in *B. subtilis*, which encodes TRAP. Here again, TRAP exerts regulatory influences across metabolic pathways, in this case between the Trp and folate pathways. TRAP not only regulates the *trp* operon

by both transcription attenuation and a translational control mechanism, it also regulates the translation of *pabAb* (required for both Trp and folate biosynthesis), *yhg* (a putative Trp transporter), and *ycbk* (encoding a protein of unknown function). Thus, Trp and folate biosynthesis are coordinated via the regulatory abilities of TRAP. An organism such as *Oceanobacillus* possesses *mtrB*, a seven-gene *trp* operon that contains *trpAb*, and a folate operon that contains *pabAb*. Thus, it seems likely that once the dual regulatory role of *mtrB* in both pathways was established, integration was further elevated in the *B. subtilis*/*B. halodurans*/*B. stearothermophilus* clade by loss of *trpAa* and reliance upon *pabAb* to form alternative complexes with either *trpAa* or *pabAa*.

Does Regulation Power Evolutionary Dynamics?

In view of the foregoing points, we offer the following broad perspective. In ancient free-living prokaryotes, *trp* structural genes had already become organized as whole-pathway operons. The selfish-operon model of operon origin promoted by Lawrence and Roth (52) might apply to these early stages. Presumably, coordinate expression from a common promoter, overlapping genes (perhaps protecting from mRNA degradation), and translational coupling (perhaps accommodating protein-protein interactions) have been of selective benefit. If, however, these are relatively weak benefits, then persistent gene scrambling may have been tolerated prior to the eventual acquisition of operons having the ultimate detail of regulation seen in the contemporary *E. coli*, *B. subtilis*, and *L. lactis.* An intermediate stage of regulation (possibly still persisting in some contemporary lineages) might have been a simplified form of transcription control involving small molecules that can bind directly to RNA and regulate attenuation. Attenuation mechanisms may have evolved in an RNA world (45), and a number of recent articles (63, 61, 82) describing the ability of small molecules to interact directly with nascent RNA suggest that this mechanism for influencing transcription might be widespread.

Aspects of regulation that may merit increased attention are the factors that influence the rate of mRNA decay. It is generally accepted that the differential stability of mRNA plays an important role in determining the steady-state levels of gene expression. Individual mRNA decay rates can vary more than 100-fold. In contrast to the level of knowledge about initiation of *trp* gene transcription, little is known about the specificity, precision, and regulatory role of mRNA decay. New capabilities for the systematic measurement of mRNA decay rates (83) should enhance our understanding of this important aspect of regulation.

One can envision that such mechanisms might have preceded the commitment of genetic material to the elaboration of regulatory proteins. Consider the relative contribution of attenuation (relatively weak) and *trpR*-mediated repression (relatively strong) in *E. coli*. Repression is designed to detect Trp, whereas attenuation is designed to detect uncharged $tTNA^{Trp}$. Under many growth conditions, the free Trp concentration in the cell may be fairly low but still sufficient to keep $tRNA^{Trp}$ largely charged. Thus, *trpR*-mediated repression is responsible for a large range of expression, and only after maximal derepression does relief from attenuation ensue. Consider also that the repressor binds not only to the *trp* operator but also to operators relevant to DAHP synthase and *trpR* itself (autoregulation). The modern whole-pathway operon systems that do possess efficient control features should be highly stable, barring any evolutionary transitions to pathogenic or symbiotic relationships. This would not preclude presumably desirable changes such as gene fusions. Simple, unregulated operons (both ancient and modern) or weakly regulated operons can be expected to be relatively unstable compared to complex, regulated operon systems that can sense a variety of different cues with a good range of sensitivity. To the extent that these deploy unlinked regulatory elements, intergenomic transfer should be relatively unlikely due to the necessity for cotransfer of unlinked genes in order to obtain the complete operon system.

FUTURE PROSPECTS FOR ELEVATED KNOWLEDGE OF Trp PATHWAY EVOLUTION

The comparative organization of the seven structural genes responsible for Trp biosynthesis has been analyzed in comprehensive detail. We have asserted that the vertical trace of descent with respect to the primary pathway can be sorted from paralogy that leads to specialized pathways and from occasional events of LGT. We have shown how relatively nonconserved contexts of flanking genes in relatively narrow organismal clades can be used to elucidate which of two evolutionary states is derived and which is ancestral. We have given examples of how the ancestral state at a given phylogenetic node can be determined.

Thus, we are beginning to get a fairly good picture of the evolutionary progressions that have taken place with respect to the organization of *trp* genes as whole-pathway operons, partial-pathway operons, and dispersed genes. However, a rationale for what driving forces exist to power the evolutionary dynamics that we can describe is not so clear. This limitation can probably be attributed to the relatively small amount of information about Trp pathway regulation that is available in the broad comparative context. To completely describe *trp* operon systems, one needs to evaluate any linked or unlinked regulatory elements that may exist. Two widely spaced organisms may have identical whole-pathway *trp* operons but may have evolved completely different control systems, or one of the two may be quite complex and the other simple. It seems significant that the current systems of *trp* operon regulation that can be described as elaborate are present in narrow bacterial clades and therefore must be of relatively recent origin. Comparative bioinformatics data to elucidate the range of regulatory mechanisms in place for *trp* operons in modern organisms is an initiative that is only beginning (60) and should be most informative.

Complexly regulated Trp systems are likely to involve the integration of Trp biosynthesis with other pathways, as has been elucidated between Trp and folate (mediated by TRAP) in *B. subtilis* or between Trp and the greater aromatic pathway (mediated by TrpR) in *E. coli*. One could envision a yet-to-be discovered metabolic relationship between Trp and serine or between Trp and histidine.

A second aspect of complexity involves the variety of multiple pathways that can exist within a single organism in which Trp or Trp intermediates can have different fates. For example,

Streptomyces coelicolor has four TrpAa/TrpAb homologues that compete to direct chorismate to the specific alternative fates of phenazine biosynthesis, antibiotic biosynthesis, siderophore (coelibactin) biosynthesis, and primary Trp substrate for protein synthesis. All of these competing systems would be expected to respond to entirely different regulatory cues. In some cases, a given *trp* gene product may be shared by more than one pathway. Larger genomes can be expected to more frequently exhibit this kind of paralogy/xenology complexity, and indeed we have seen examples for the Trp pathway in large-genome organisms such as *Nostoc* sp., *Pseudomonas aeruginosa*, and *Streptomyces coelicolor.*

In this article, a strong foundation has been developed that should help guide the selection of key organisms for studies designed to gain insight into how Trp pathway regulation is related to the driving forces of evolution.

APPENDIX

Analysis of Raw DNA Sequence Data

Raw DNA contig sequences available from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgemome.html) and TIGR unfinished microbial genomes database (http://www.tigr.org/tdb/ufmg/) were screened with the built-in Blast service. The protein sequences from GenBank were used as query entries. The Blast 2.0 (5) and the open reading frame finder (ORF Finder) offered by NCBI were used to locate open reading frames and to confirm the similarity search result of the raw sequence.

Deduced amino acid sequences were analyzed for N-terminal signal sequences and transmembrane domains with Psort (http://psort.ims.u-tokyo.ac.jp/) (64).

Hidden Markov model and Prosite pattern search. Multiple alignments were obtained with the ClustalW program (78) included in the BioEdit (version 5.0.9) multiple alignment tool (33). A hidden Markov model based upon a multiple sequence alignment of known TrpC sequences was generated by version 2.2g of the HMMER program (22). A Prosite-like regular expression pattern was generated manually, and this hidden Markov model and Prosite pattern were further searched against the genomes that are missing *trpC*.

16S rRNA Tree Construction

16S rRNA subtrees were obtained from the Ribosomal Datebase site (http://rdp.cme.msu.edu/html/) (55).

DNA Composition

The GC percentages for individual genes were computed with the GEECEE program, which was written by R. Bruskiewich at the Sanger Centre (Cambridge, United Kingdom). The whole-genome GC value was obtained from the codon usage database (http://www.kazusa.or.jp/codon/) (65).

Fusion Protein and Linker Region Analyses

All the fusion protein sequences from the GenBank and NCBI Microbial Genomes Blast databases (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html) werescreened by use of Blast (5) service. The known fusion protein sequences were used as query entries. A multiple alignment was obtained by input of single-domain and fusion protein sequences into the ClustalW (78) program (version 1.4). The linker region was defined by comparing the multiple sequence alignment of fusion proteins and monofunction proteins. Then the conserved domain database result (56) was used as the reference guide to find the boundary of the fusion protein (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

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