

Dispersal and regulation of an adaptive mutagenesis cassette in the bacteria domain

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ABSTRACT

Recently, a multiple gene cassette with mutagenic translation synthesis activity was identified and shown to be under LexA regulation in several proteobacteria species. In this work, we have traced down instances of this multiple gene cassette across the bacteria domain. Phylogenetic analyses show that this cassette has undergone several reorganizations since its inception in the actinobacteria, and that it has dispersed across the bacterial domain through a combination of vertical inheritance, lateral gene transfer and duplication. In addition, our analyses show that LexA regulation of this multiple gene cassette is persistent in all the phyla in which it has been detected, and suggest that this regulation is prompted by the combined activity of two of its constituent genes: a polymerase V homologue and an alpha subunit of the DNA polymerase III.

INTRODUCTION

Adaptive mutation in bacteria has become a field of increasing interest in the last years. Also termed stationary-phase or stress-induced mutation (1), adaptive mutation concerns the mechanisms by which bacteria increase their mutation rate when placed under non-lethal selective conditions, thereby spontaneously generating mutations that relieve the selective pressure. Adaptive mutation is of special interest in microbiology because it has a broad influence on the mechanisms of evolution, and particularly on those taking place in closed and stressful media such as the hosts of bacterial pathogens. It is therefore of importance to the study of pathogen evolution inside a host, and more specifically to the analysis of antibiotic

resistance generation and of bacterial co-evolution with the host immune response.

A major mechanism in adaptive mutation is translesion synthesis (TLS). Mediated by error-prone or lesion bypass polymerases, TLS allows the cell to replicate past a variety of DNA lesions and distortions, thereby promoting survival under endogenous or environmental insults, but also drastically increasing the stationary-phase mutation rate (1). Members of the recently discovered Y family of DNA polymerases, such as *Escherichia coli* polymerases IV (encoded by the *dinB* gene) and V (encoded by the *umuD* and *umuC* genes), have been shown to be error prone and poorly processive, lying at the core of TLS in most organisms (2). Indeed, Y family polymerases are present in all live domains and have been linked to a range of mutagenic activities: from adaptive mutagenesis in bacteria (3) to genetic instability in human cancer (4).

Well before their true nature was established, both *E.coli* *dinB* and *umuDC* products have already been linked to the SOS response (5). First described in *E.coli*, the SOS system is a global response mechanism against DNA damage that in *E.coli* regulates up to 40 genes involved in DNA repair and cell survival (6,7). Induced by single-stranded fragments of DNA generated by either DNA damage-mediated replication inhibition or enzymatic processing of broken DNA ends (8), the SOS response is governed by the LexA and RecA proteins, both of which are also members of this regulatory network. In normal conditions, LexA binds to operator sites of regulated genes and effectively represses their expression. Conversely, in the advent of DNA damage single-stranded DNA (ssDNA) fragments bind and activate the RecA protein, which acquires coprotease activity and promotes the autocatalytic cleavage of LexA (9). Cleaved LexA is unable to bind DNA, leading to the unrepressed expression of SOS genes. Once DNA damage has been addressed, ssDNA concentration falls and both RecA and LexA regain their usual

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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conformation, repressing again the system. The LexA protein is widespread among bacteria and several distinct LexA-binding sites have been reported in different phylogenetic groups. *E. coli* LexA, for instance, binds a 16 bp consensus sequence (CTGTN₈ACAG) (5) that is also found in most gamma and beta proteobacteria (10). Similarly, Gram-positive bacteria LexA has been shown to bind a palindromic motif with consensus GAACN₄GTTY (11), which is very similar to that observed in cyanobacteria and green non-sulfur bacteria (12,13). Additional LexA-binding sites have been defined in the alpha proteobacteria (GTTCN₇GTTC) (14,15), the Xanthomonadales (TTAN₆TACTA) (16), *Fibrobacter succinogenes* (TGCNCN₄GTGCA) (17) and the delta proteobacteria *Myxococcus xanthus* (CTRHAMRY-BYGTTTCAGS) (18) and *Bdellovibrio bacteriovorus* (TTACN₃GTAA) (19).

Recently, a multiple gene cassette encoding two error-prone polymerases was described in *Pseudomonas putida* and homologs of this same cassette were identified in several gamma, beta and alpha proteobacteria species (20). The original gene cassette in *P. putida* encoded a copy of the LexA repressor (PP3116) recognizing a cyanobacteria-like LexA-binding sequence, a protein annotated in *P. putida* as Sula (PP3117), a *dinB* homolog (PP3118) and an alpha subunit of the DNA polymerase III (PP3119). This cassette was shown to be a DNA-damage inducible operon, self-regulated by its own encoded LexA protein, and homologs of this full *P. putida* gene cassette were identified in the genomes of *Pseudomonas syringae*, *Pseudomonas fluorescens* and all the sequenced *Xanthomonadaceae* (20). In addition, three-gene cassette derivatives lacking the *lexA* gene were also found in the close *Pseudomonas aeruginosa*, the beta proteobacterium *Ralstonia solanacearum* and many alpha proteobacteria species such as *Agrobacterium tumefaciens* (20). Interestingly, all the reported cassette instances harbored also an adapted LexA-binding site on the promoter region of their first gene, and both DNA-damage inducibility and LexA binding were experimentally confirmed for cassette homologs in *A. tumefaciens*, *P. aeruginosa*, *Sinorhizobium meliloti* and *Xanthomonas campestris* (20). Given the aforementioned divergence in LexA-binding sequences among these bacterial groups, the persistence of LexA regulation in all these species hinted at a positive pressure towards LexA control of this multiple gene cassette.

Later work on *Caulobacter crescentus* (21) confirmed that its three-gene cassette homolog was also a DNA-damage inducible operon, negatively regulated by *C. crescentus* LexA protein, and named its constituents as *imuA*, *imuB* and *dnaE2* (PP3117, PP3118 and PP3119 homologs, respectively). Furthermore, experimental assays demonstrated that the *imuA-imuB-dnaE2* cassette is responsible for most DNA-damage induced mutations in *C. crescentus*, and is required for error-prone processing of DNA lesions in this species (21). In addition, phylogenetic analyses revealed that the cassette *dnaE2* gene was related to *Mycobacterium tuberculosis dnaE2* gene, whose product had been shown previously to mediate SOS mutagenesis in this species. Further experiments confirmed that the *imuB* and *dnaE2* gene products of *C. crescentus* cooperated in lesion bypass, with a mutational signature different from that observed previously in the *E. coli umuDC* system. The fact that

C. crescentus and all the sequenced alpha proteobacteria, in which the presence of the *imuA-imuB-dnaE2* cassette is ubiquitous, lack an *umuDC* TLS system suggested that this gene cassette might be playing a TLS role in these species. This line of reasoning was strengthened with the identification of split cassette homologues in other species lacking an *umuDC* system, such as the Planctomycetes *Rhodopirellula baltica* or the Actinomycetales *M. tuberculosis* and *Propionibacterium acnes*, although the first gene of these split cassette instances in the actinobacteria, which we will here term *imuA'*, could not be shown to be an homolog of its proteobacteria equivalent *imuA*.

More recent work has also identified a DNA-damage inducible, LexA-regulated *imuA-imuB-dnaE2*-like cassette in the delta proteobacterium *B. bacteriovorus* (19), and has shown that its *imuA* gene, although annotated as and sharing partial homology with *recA*, does not *trans*-complement a *recA* defective *E. coli* strain. To further elucidate the nature and evolution of this multiple gene cassette, here we have conducted an extensive database search of *imuA*, *imuA'*, *imuB* and *dnaE2* homologs across the bacteria domain, and we have made use of robust phylogenetic methods to infer the evolutionary history of this gene cassette, revealing its dispersal through duplications, vertical inheritance and lateral gene transfer (LGT), and its continued regulation by and influence on the SOS system.

MATERIALS AND METHODS

Gene, protein and genomic sequences

Protein and DNA sequences for *lexA*, *imuA'*, *imuA*, *imuB* and *dnaE2* annotated homologs and their respective promoters were downloaded from NCBI GenBank, TIGR Comprehensive Microbial Resource and JGI Integrated Microbial Genomes database resources after identification of homologs by the on-site database textual or BLAST (22) search tools. Additional *lexA*, *imuA'*, *imuA*, *imuB* and *dnaE2* DNA sequences from homologs in unfinished microbial genomes were downloaded from either JGI Integrated Microbial Genomes or TIGR Unfinished Microbial Resource after their identification through these resources own BLAST services, and DNA sequences were translated into their corresponding amino acid sequences with the EditSeq v5.0 program (*DNAStar*). Complete and incomplete genome assemblies for the organisms harboring *imuA'*, *imuA*, *imuB* and *dnaE2* homologs were downloaded also from the aforementioned resources.

Identification of LexA regulatory motifs

LexA regulatory motifs in the promoter of *lexA*, *imuA'*, *imuA*, *imuB* and *dnaE2* genes were searched using RCGScanner v2.1 (10), a consensus-building software for the prediction of regulatory motifs, in those species with known or inferred LexA-binding sequences. Taking advantage of the fact that *lexA* is a self-regulated gene, undescribed LexA regulatory motifs were identified by first looking for putative dyad motifs in the promoter region of *lexA* using custom MsWord macro routines to detect palindromic and direct repeat motifs with varying number of mismatches and spacer lengths. Candidate motifs were then

sought in the promoter regions of *recA* and *imuA'/imuA* or *dnaE2* genes with EditSeq's Find function allowing for ambiguity and, if found, the significance of these findings was independently corroborated using the motif discovery tool MEME (23) through its web-based interface at the San Diego Supercomputing Center.

Phylogenetic analyses

Alignments of protein sequences were carried out using a combined procedure to improve alignment quality. Protein sequences were first aligned through CLUSTALW v1.83 (24) using Gonnet matrices and default [10], 25 and 5 gap-opening penalties for the multiple alignment stage, thus generating three different alignments. These three slightly different alignments, together with a local alignment generated by T-COFFEE Lalign method, were integrated as libraries into T-COFFEE v1.37 (25) for optimization. T-COFFEE is an alignment tool for the optimization of parametric consistency in multiple alignments that draws on different alignments to reduce the impact of initial errors in greedy progressive alignment methods. The optimized alignment was then visually inspected with BioEdit v5.0.9 (26) and submitted to Gblocks v0.91b (27) with the half-gaps setting and otherwise default parameters to select conserved positions and discard poorly aligned and phylogenetically unreliable information.

Phylogenetic analyses of the T-COFFEE optimized alignments were carried out using both MrBayes v3.1.1 for Bayesian (28) and PHYML v2.4.1 (29) for maximum-likelihood (ML) inference of tree topologies. In both cases, a mixed four-category gamma distributed rate plus proportion of invariable sites model [invgamma] was applied and its parameters were estimated independently by each program. Four independent MrBayes Metropolis-Coupled Markov Chain Monte Carlo runs were carried out with four independent chains for 10^6 generations, and 1000 bootstrap replicates were used for ML inference with PHYML. The resulting phylogenetic trees were plotted with TreeView v1.6.6 (30) and enhanced for presentation using CorelDraw Graphic Suite v12 (Corel Corporation).

RESULTS AND DISCUSSION

Identification of additional instances of the *imuA-imuB-dnaE2* cassette

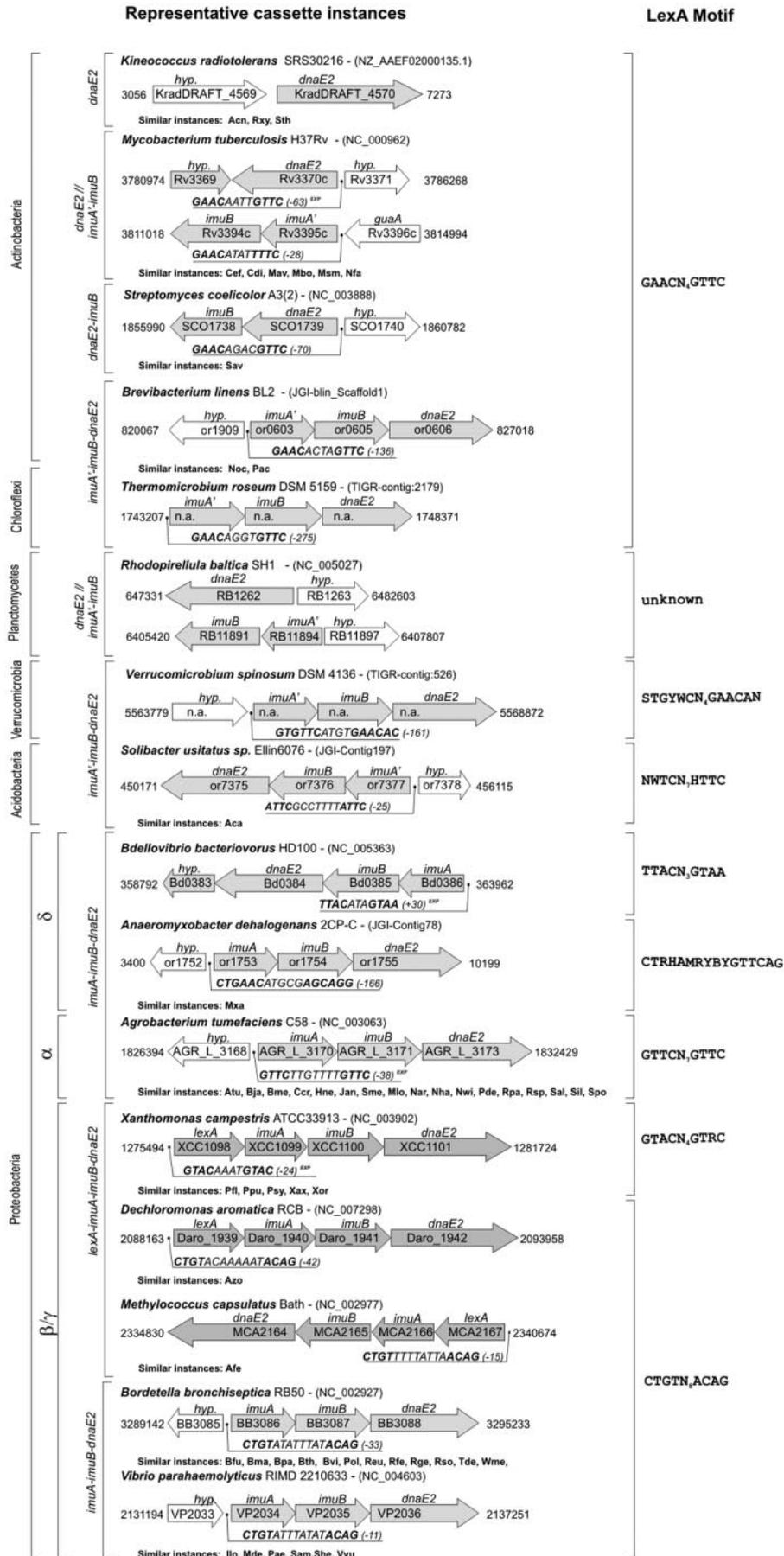
Using BLAST and PSI-BLAST searches with the products of each of the cassette genes as queries, homologs of this multiple gene cassette were located in most bacterial phyla, ranging from the actinobacteria to the proteobacteria. Cast in the light of the accepted branching order of bacteria, as established by 16S RNA (31), RecA (32) and protein signature phylogenies (33), these findings reveal either a progressive accretion or fragmentation of the *imuA-imuB-dnaE2* cassette in the actinobacteria, punctuated by several genetic reorganizations that gave rise to a range of different cassette configurations.

As it can be seen in Figure 1, the simplest instance of an *imuA-imuB-dnaE2* cassette constituent can be traced back to some actinobacteria, such as *Kineococcus radiotolerans*, *Symbiobacterium thermophilum* or *Actinomyces naeslundii*, in which only the *dnaE2* gene can be found. Close

actinobacteria, like the Streptomycineae, show evidence of a two-gene cassette consisting of the *dnaE2* and a *dinB* homolog which we here ascribe to *imuB*. On the other hand, several actinobacteria present split cassette homologs in which the *dnaE2* gene is isolated while two additional genes, the RecA homolog here labeled as *imuA'* and *imuB*, make up a two-gene cassette. This is the case of the previously reported *M.tuberculosis*, but also of all the sequenced Mycobacteriaceae, Corynebacterineae and of *Nocardia farcinica*. Finally, instances of an *imuA'-imuB-dnaE2* cassette can also be found within the actinobacteria. *P.acnes*, *Nocardioides* sp. and *Brevibacterium linens* all possess a three-gene cassette, and this is very likely the layout of the *imuA'-imuB-dnaE2* cassette that emerged from the actinobacteria into subsequent phyla.

Leaving the actinobacteria, an *imuA'-imuB-dnaE2* like cassette can be detected in several bacterial phyla preceding the proteobacteria, which is all the more remarkable due to the scant representation most of these phyla share in the sequenced microbial genomes databases. An *imuA'-imuB-dnaE2* like cassette is found in the Chloroflexi *Thermomicrobium roseum*, the Planctomycetes *R.baltica*, the Verrucomicrobia *Verrucomicrobium spinosum* and the two partially sequenced acidobacteria species (*Acidobacterium capsulatum* and *Solibacter usitatus*). In all these instances, the *imuA'-imuB-dnaE2* cassette presents the same configuration and apparently reflects a pattern of vertical transmission from a common ancestor with the actinobacteria that ultimately leads to the delta proteobacteria, where *imuA-imuB-dnaE2* cassette instances can be identified in *B.bacteriovorus* and *Anaeromyxobacter dehalogenans*. Somewhere along this line, however, the *imuA'* gene must have undergone drastic changes, involving either extensive mutation or, most probably, a partial or complete substitution by recombination that ultimately led to the *imuA* gene observed in the proteobacteria. A reliable phylogeny of the *imuA'* and *imuA* genes cannot be reconstructed, since alignments generated with both sequences present almost no conserved positions, backing up the previous suggestion (21) that *imuA* and *imuA'* are not homologs, but could be functional analogs. Moreover, separate phylogenies of *imuA* and *imuA'* become only consistent when restricting the phylogeny to well-defined groups such as the actinobacteria or the proteobacteria (data not shown), making it impossible to precisely define the point at which the aforementioned recombination event took place.

The *imuA-imuB-dnaE2* configuration found in the delta proteobacteria is also maintained in the alpha proteobacteria, where cassette instances can be found in all completely sequenced genomes and even in some plasmids (e.g. *A.tumefaciens*). Similar three-gene cassettes are also found in several gamma and beta proteobacteria species (e.g. *Shewanella oneidensis*, *Vibrio parahaemolyticus*), although cassette prevalence is not so regular in these bacterial classes. Neither *imuA-imuB-dnaE2* cassettes, nor any of their constituent genes, can be found, for instance, in the *Enterobacteriaceae* and the *Pasteurellaceae*, although both are richly sampled families in terms of available genome sequences. In addition, both gamma and beta proteobacteria present several instances of *lexA-imuA-imuB-dnaE2* cassettes. These include the previously described four-gene cassettes of



P.putida, *P.fluorescens*, *P.syringae* and the *Xanthomonadaceae* (20), together with additional instances in *Acidithiobacillus ferrooxidans*, *Methylococcus capsulatus* and the beta proteobacteria *Dechloromonas aromatica*, *Thiobacillus denitrificans* and *Azoarcus* sp.

LexA regulation of *imuA-imuB-dnaE2* cassettes

As mentioned above, a striking characteristic of the previously identified *imuA-imuB-dnaE2* and *lexA-imuA-imuB-dnaE2* cassettes in the alpha, gamma and beta proteobacteria was their persistent regulation by their host (or their own encoded) LexA protein in spite of the drastic differences between the regulatory motifs of these LexA proteins (10,15,20). Together with the existence of *lexA-imuA-imuB-dnaE2* cassettes, in which a *lexA* gene seems to be specifically set to control the adjacent *imuA-imuB-dnaE2* genes, and the complete absence of cassette instances in those phyla and classes lacking a *lexA* gene (e.g. Epsilon Proteobacteria), this suggested that LexA regulation of *imuA-imuB-dnaE2*-like cassettes ought to be markedly beneficial for their bacterial hosts. Having established that the presence of *imuA-imuB-dnaE2* cassettes was not limited to the proteobacteria, and given that a new cassette instance in *B.bacteriovorus* (19) had also been shown to be regulated by LexA with yet another markedly divergent LexA-binding motif (TTACN₃GTAA), we set about to examine LexA regulation in all the here-identified cassette instances.

Interestingly, even though no significant occurrences of the Gram-positive LexA-binding motif (11) can be located upstream of *dnaE2* genes in those species (*K.radiotolerans*, *S.thermophilum* and *A.naeslundii*) presenting only this constituent of the *imuA'-imuB-dnaE2* cassette, high-scoring and well-placed Gram-positive LexA-binding sites can already be found preceding the *dnaE2* gene in the *dnaE2-imuB* cassettes of the Streptomycineae. As shown in Figure 1, a similar pattern of LexA regulation is conserved also in those actinobacteria presenting split cassette instances, with an *imuA'-imuB* tandem and an isolated *dnaE2* gene. In these cases, of which *M.tuberculosis* is a well-known representative, apparent LexA regulation does not only concern the isolated *dnaE2* gene [which has been experimentally confirmed, (34)], but extends also to the *imuA'-imuB* tandem. Even though in some cases, like *N.farcinica*, evidence of LexA regulation can be found only in the *imuA'-imuB* tandem, the systematic regulation of at least one of the cassette components in all these species suggests that it is their concerted activity that creates a positive pressure towards LexA regulation. In this context, it should be stressed that a combined activity of the

imuB and *dnaE2* genes of *C.crescentus* has already been singled out as the source of most TLS mutagenic activity in this species (21), and that unregulated TLS activity mediated by *dinB* has been shown previously to yield mutator phenotypes in *E.coli* (35), resulting in lowered adaptative fitness.

As expected, LexA regulation is maintained in most actinobacteria presenting full *imuA'-imuB-dnaE2* cassettes. The sole exception concerns *Rubrobacter xylanophilus*, but this bacterium does not present a Gram-positive LexA-binding motif upstream of its *lexA* gene and its *lexA* gene sequence shows substantial divergence from that of other actinobacteria, suggesting that it may have evolved separately to recognize a new LexA-binding motif. Outside the actinobacteria, a Gram-positive LexA-binding motif can be identified upstream of both *T.roseum lexA* and *imuA'* genes, suggesting that, in addition to the Dehalococcoidetes (13), this is also the LexA-binding motif of the Thermomicrobiales. Interestingly, a derivative of the Gram-positive LexA-binding motif with reminiscences of the one seen in *E.coli* (STGYWCAWNYGAACAN) can also be found upstream of *V.spinosum lexA*, *recA* and *imuA'* genes, suggesting that this is the LexA-binding sequence in this species and that the *imuA'-imuB-dnaE2* cassette is regulated by *V.spinosum* LexA. A similar situation is found also in the acidobacteria, where NWTCN₇HTTC direct repeat motifs (close to the one associated with the alpha proteobacteria) (15) can be located upstream of the *lexA* and *imuA'* genes in both species. This is all the more remarkable given that *A.capsulatum* presents two instances of the *imuA'-imuB-dnaE2*, and the aforementioned LexA-binding motif is present upstream of both *imuA'* genes.

LexA regulation is also preserved in the delta proteobacteria. Besides the *imuA-imuB-dnaE2* cassette already shown to be LexA regulated and DNA damage inducible in *B.bacteriovorus* (19), derivatives of the *M.xanthus* LexA-binding sequence (18) can also be found upstream of *A.dehalogenans lexA*, *recA* and *imuA* genes. Interestingly, in *M.xanthus*, where only the *dnaE2* gene can be found, there is no evidence of a LexA-binding sequence upstream of it, suggesting again that it is the combined presence of *imuB* and *dnaE2* in a bacterial genome that prompts LexA regulation. As mentioned earlier, the presence of *imuA-imuB-dnaE2* cassettes among the alpha proteobacteria is pervasive, with some species harboring up to three copies of this cassette (e.g. *A.tumefaciens*) and frequent evidence of plasmid dissemination. Consistent with this widespread distribution, LexA regulation and DNA-damage induction of the *imuA-imuB-dnaE2* cassettes have been reported in

Figure 1. Schematic representation of representative cassette configurations and similar instances found in complete and incomplete genome sequences. All positions are relative to genome/contig start position. n.a. and (BLAST) indicate non-annotated cassette instances of incomplete genomes located through BLAST. EXP denotes experimentally verified LexA-binding motifs. Bacterial name abbreviations for similar instances are as follows: Aca—*A.capsulatum*, Acn—*A.naeslundii*, Afe—*A.ferrooxidans*, Atu—*A.tumefaciens*, Azo—*Azoarcus* sp., Bfu—*Burkholderia fungorum*, Bja—*Bradyrhizobium japonicum*, Bma—*Burkholderia mallei*, Bme—*Brucella melitensis*, Bpa—*Bordetella parapertussis*, Bth—*Burkholderia thailandensis*, Bvi—*Burkholderia vietnamiensis*, Ccr—*C.crescentus*, Cdi—*Corynebacterium diphtheriae*, Cef—*Corynebacterium efficiens*, Hne—*Hyphomonas neptunium*, Ilo—*I.loihiensis*, Jan—*Jannaschia* sp., Mav—*Mycobacterium avium*, Mbo—*Mycobacterium bovis*, Mde—*M.degradans*, Mlo—*Mesorhizobium loti*, Msm—*Mycobacterium smegmatis*, Mxa—*M.xanthus*, Nar—*Novosphingobium aromaticivorans*, Nfa—*N.farcinica*, Nha—*Nitrobacter hamburgensis*, Noc—*Nocardioideis* sp., Nwi—*Nitrobacter winogradskyi*, Pac—*P.acnes*, Pae—*P.aeruginosa*, Pde—*Paracoccus denitrificans*, Pfl—*P.fluorescens*, Pol—*Polaromonas* sp., Ppu—*P.putida*, Psy—*P.syringae*, Reu—*Ralstonia eutropha*, Rfe—*Rhodospirillum rubrum*, Rge—*Rubrivivax gelatinosus*, Rpa—*Rhodospseudomonas palustris*, Rsp—*Rhodobacter sphaeroides*, Rso—*R.solanacearum*, Rxy—*R.xylanophilus*, Sal—*Sphingopyxis alaskensis*, Sam—*Shewanella amazonensis*, Sav—*Streptomyces avermitilis*, She—*Shewanella* sp., Sil—*Silicibacter* sp., Sme—*S.meliloti*, Spo—*Silicibacter pomeroyi*, Sth—*S.thermophilum*, Tde—*T.denitrificans*, Vvu—*Vibrio vulnificus*, Wme—*Wautersia metallidurans*, Xax—*Xanthomonas axonopodis* and Xor—*Xanthomonas oryzae*. Additional information for each cassette instance is provided in Supplementary Table S1, which is included as Supplementary Data.

A.tumefaciens, *C.crescentus* and *S.meliloti* (20,21), and the corresponding LexA-binding motifs have been found upstream of *imuA* in other alpha proteobacteria species (15).

Concerning the gamma and beta proteobacteria, LexA regulation has been reported (10) or is apparent in all the identified instances of the *imuA-imuB-dnaE2* cassette. It is interesting to note, however, that the previously reported (20) LexA-binding sequence of *lexA-imuA-imuB-dnaE2* cassettes (GTACN₄GTGC) is not present in all the here-identified four-gene cassette instances. *A.ferrooxidans*, *T.denitrificans*, *D.aromatica* and *Azoarcus* all present an *E.coli*-like LexA-binding sequence upstream of their cassette *lexA* gene which, in contrast to the *Pseudomonadaceae* and the *Xanthomonadaceae*, is the sole *lexA* gene in their genome.

Reorganization and lateral gene transfer of the *imuA-imuB-dnaE2* cassette

Even though the overall distribution of the *imuA-imuB-dnaE2* cassette is in approximate agreement with the established phylogeny of bacteria, its broad dispersal is punctuated by noticeable absences (e.g. cyanobacteria, epsilon proteobacteria) and seems to be intimately linked to the LexA network, as evidenced by its persistent regulation, the presence of four-gene cassettes including a *lexA* gene and the absence of cassettes in all classes and phyla lacking a *lexA* gene. Moreover, the identification of several *imuA-imuB-dnaE2* cassette duplication events (e.g. *A.capsulatum*, *A.tumefaciens*) and its recurring existence in plasmids (e.g. *R.solanacearum*, *S.meliloti*) had suggested previously that the evolution of this cassette might have also been shaped by other factors, such as LGT. Therefore, to ascertain whether there existed relevant discrepancies in the particular phylogeny of the *imuA-imuB-dnaE2* cassette, a rigorous phylogenetic analysis of this multiple gene cassette was conducted. Owing to the aforementioned lack of homology between *imuA* and *imuA'*, these sequences were discarded for the analysis. Similarly, optimized alignments of *imuB* sequences following our astringent protocols yielded too few conserved positions (below 30) for a sound phylogenetic analysis over the whole bacteria domain. Therefore, and given that *imuB* genes were also absent in some of the identified cassette instances (e.g. *K.radiotolerans*), the phylogenetic analysis was carried out using the *dnaE2* gene product sequence, which was both the most phylogenetically informative of the cassette gene sequences (623 conserved positions) and also the only one providing full coverage among the identified cassette instances. Furthermore, to validate and reinforce the phylogenetic approach, the analysis was also conducted on the *dnaE1* gene product sequence in all the species studied. The *dnaE1* gene, corresponding to the normal (non-cassette) alpha subunit of the DNA polymerase III, is similar in length to the *dnaE2* gene and ought to be subjected to similar structural constraints, thus providing a reliable contrast to the evolution of the *dnaE2* gene and facilitating the identification of phylogenetic artifacts, such as those due to fast-clocked evolution.

As it can be seen in Figure 2, the DnaE1 sequence produces a tree that is in broad agreement with the established phylogenies (31–33). A natural cluster groups all the actinobacteria except those (*R.xylanophilus* and *S.thermophilum*)

that have already been suggested to be part of to-date undefined groups of actinobacteria (36–37). Both these species are positioned at the exit of the actinobacteria clade, intermingling with the intermediate phyla (Planctomycetes, green non-sulfur bacteria, acidobacteria and Verrucomicrobia) that then lead to the proteobacteria, in which the natural branching order (delta, alpha, beta and gamma) can be observed. In agreement with previous phylogenies (38,39), the *Xanthomonadaceae* branch earlier than gamma and beta proteobacteria, suggesting that they constitute a separate group of early gamma/beta proteobacteria.

A similar story is told by the DnaE2 sequence tree up to the branching point of beta and gamma proteobacteria (Figure 3). A main cluster encompasses most of the actinobacteria, displaying a branching order in which an *imuA'-imuB-dnaE2* cassette configuration leads to split cassette instances and thus suggesting that the later originated following a genetic reorganization of the former. In this context, the persistent regulation of one or both members of the split cassettes suggests again a positive pressure towards LexA regulation. A separate group with long-branch lengths takes in those species purporting only a *dnaE2* gene (*S.thermophilum*, *R.xylanophilus*, *K.radiotolerans* and *T.roseum*). The *Streptomycetaceae*, which display a unique *dnaE2-imuB* cassette configuration, also fall in this last group. Even though LGT events could be invoked to explain this clustering, its origin seems to be a long-branch attraction artifact due to the substantial divergence in these sequences, a fact that would be in agreement with a period of extensive genetic reorganization that led from three-gene *imuA'-imuB-dnaE2* cassettes to the isolated *dnaE2* and tandem *dnaE2-imuB* configurations seen in these species.

In contrast, the consistent placement of the Planctomycetes *R.baltica* deep within the proteobacteria cluster is in clear disagreement with both the established and the DnaE1 phylogenies. This, together with the fact that *R.baltica* possesses a split cassette with *imuA* and *imuB* genes (RB11894 and RB11891) that present high identity with *P.putida imuA* and *imuB* (instead of actinobacteria *imuA'* and *imuB*), strongly suggests that *R.baltica* acquired its *imuA-imuB-dnaE2* cassette through LGT from an ancestor of gamma and beta proteobacteria and later reorganized it into a split cassette with an *imuA-imuB* tandem and an isolated *dnaE2* gene. In this respect, it should be noted that the clustering together of all *imuA-imuB-dnaE2* cassette instances that are located in plasmids in the alpha proteobacteria strongly suggest that there has been extensive plasmid dissemination of the *imuA-imuB-dnaE2* cassette in this bacterial class. In the light of this, and given the close placement of *R.baltica* in the DnaE2 tree (between alpha and gamma/beta proteobacteria), plasmid dissemination could certainly be the mechanism for the observed LGT event.

Duplication of the *lexA-imuA-imuB-dnaE2* cassette

Another major source of disagreement between the DnaE2 and DnaE1 trees concerns the branching point of gamma and beta proteobacteria. While the DnaE1 tree supports a view that is in close agreement with the established phylogenies, the DnaE2 tree generates three separate clusters with low support values. Two of these clusters correspond,

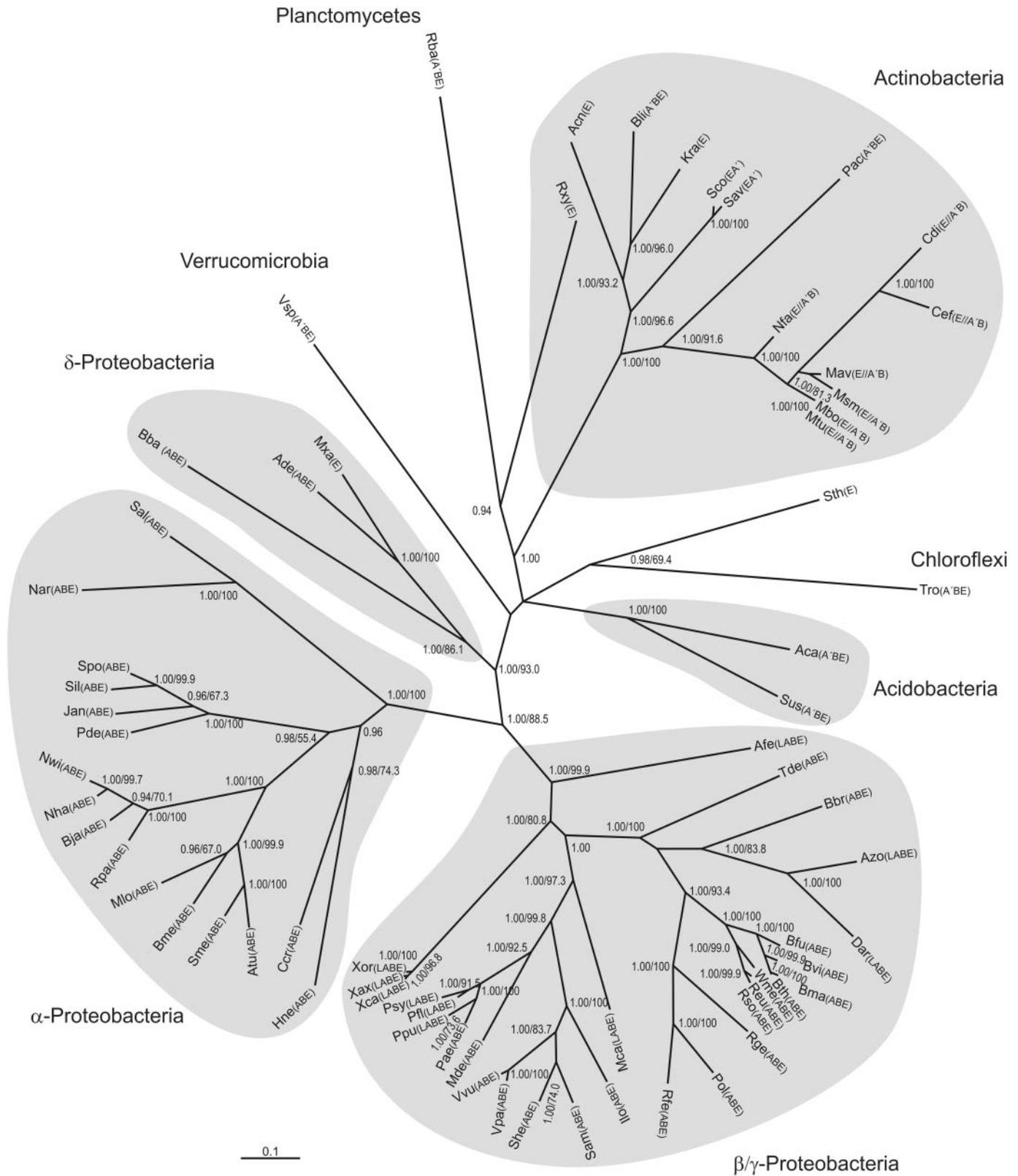
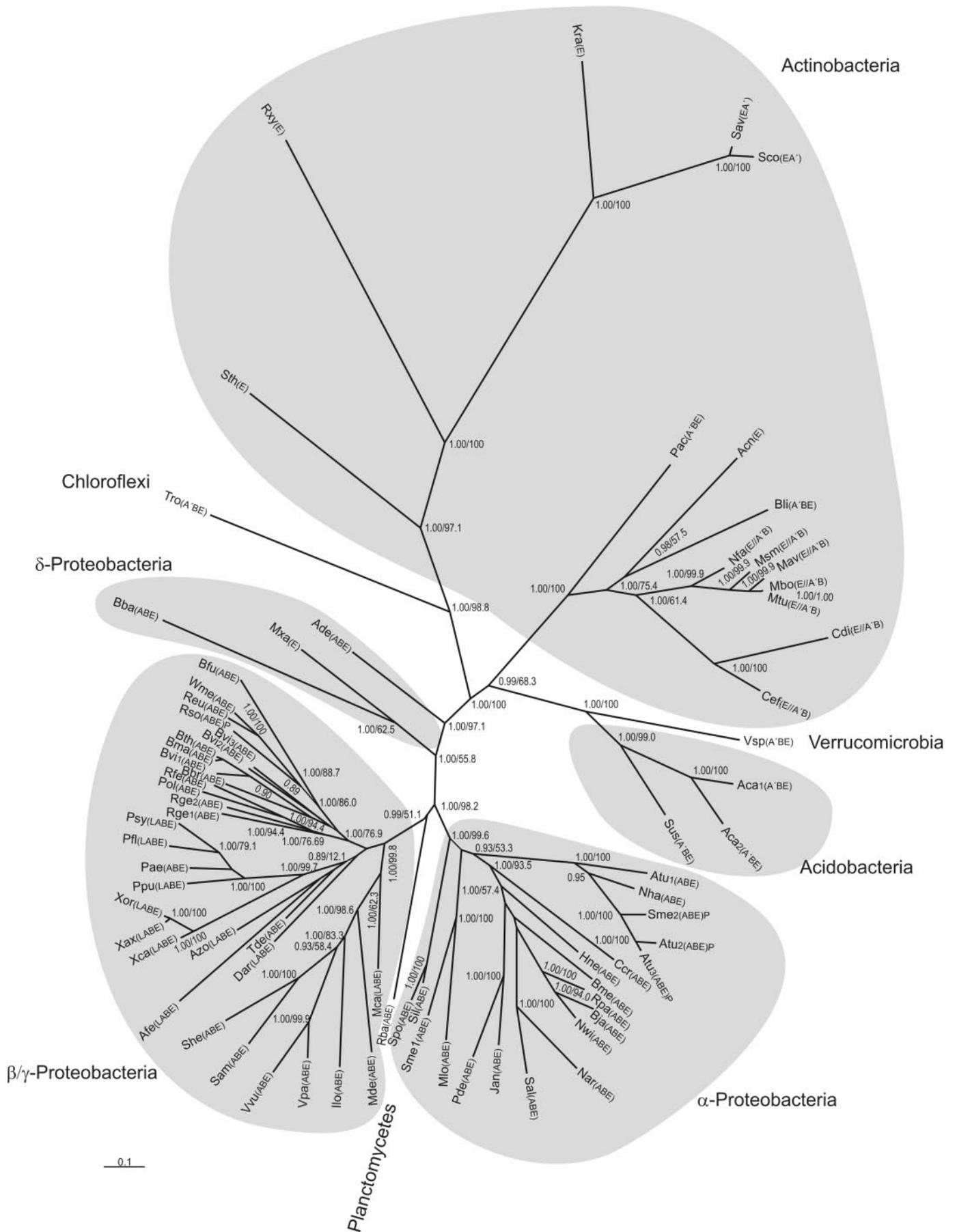


Figure 2. Unrooted DnaE1 sequence tree. Shaded areas correspond to established phylogenetic groups. Branch support values below 0.85 or 50% for Bayesian or ML inference phylogenetic analyses, respectively, are not shown. Cassette configurations are shown using constituent genes abbreviations: L—*lexA*, A'—*imuA*, A—*imuA*, B—*imuB*, E—*dnaE2*. Name abbreviations not used in Figure 1 are as follows: Bba—*Bacteriovorus*, Bbr—*Bordetella bronchiseptica*, Dar—*D. aromatica*, Kra—*K. radiotolerans*, Mca—*M. capsulatus*, Sco—*Streptomyces coelicolor*, Sus—*S. usitatus* sp., Tro—*T. roseum*, Vpa—*V. parahaemolyticus* and Vsp—*V. spinosum*. Additional information for each *dnaE1* locus is provided in Supplementary Table S1, which is included as Supplementary Data.



roughly, to the beta and gamma proteobacteria classes, while the third groups together nearly all instances of the *lexA-imuA-imuB-dnaE2* cassette instances, intermingling beta and gamma proteobacteria species. Owing to the limited resolution of the DnaE2 tree at this level, it is difficult to infer an accurate account on the evolutionary history of the cassette at this point. LGT of a primordial four-gene cassette could certainly be invoked to explain the observed data, but repeated LGT events and *ad hoc* deletions would be still required in the most plausible LGT scenario, which cannot be supported solely on the available phylogenetic data. In contrast, several facts point to an ancestral duplication of the *lexA-imuA-imuB-dnaE2* cassette, which may have then led through vertical inheritance and several deletions to the observed distribution of three- and four-gene cassettes among gamma proteobacteria. The consistent clustering of *Xanthomonadaceae* and *Pseudomonadaceae*, for instance, is reminiscent of a vertical inheritance pattern with missing intermediate gamma proteobacteria groups. Moreover, the fact that it is only in these two families that the *lexA* gene of the *lexA-imuA-imuB-dnaE2* cassette presents a markedly divergent LexA-binding motif (GTACN₄GTGC) not found in the other four-gene cassettes strongly suggests a common origin. Interestingly, *lexA* duplication has already been suggested in the *Xanthomonadaceae* (39), thus giving further support to the duplication hypothesis. In fact, a phylogeny of the LexA protein (data not shown) also clusters together both *Xanthomonadaceae* LexA proteins and one of the *Pseudomonadaceae* LexA proteins in a separate branch, while the remaining *Pseudomonadaceae* LexA protein clusters naturally with *Microbulbifer degradans* in the gamma proteobacteria cluster, thus suggesting again an ancestral duplication of *lexA*, and its attached cassette, at the root of the gamma and beta proteobacteria.

Taken together, thus, the results from the DnaE1, DnaE2 and LexA sequence trees support a scenario involving the duplication of the full *lexA-imuA-imuB-dnaE2* cassette. The proposed scenario, outlined in Figure 4, places the formation of the original *lexA-imuA-imuB-dnaE2* cassette in an ancestor of the gamma and beta proteobacteria, following a genomic reorganization that brought together the extant *imuA-imuB-dnaE2* cassette and *lexA* gene already seen in earlier proteobacteria. Drawing from the available data in the DnaE1 tree, which places most *lexA-imuA-imuB-dnaE2* cassette harboring species at the root of gamma and beta proteobacteria, this original *lexA* gene had already evolved or was on the way to evolve recognition of an *E.coli*-like motif. Shortly after, or during the own reorganization event, this *lexA-imuA-imuB-dnaE2* cassette underwent a duplication, resulting in two copies of this four-gene cassette that for the sake of clarity we will here label as *lexA¹-imuA¹-imuB¹-dnaE2¹* and *lexA²-imuA²-imuB²-dnaE2²*. Following duplication, the two cassette copies started to diverge, leading in their *lexA* gene to the full emergence of distinct LexA-binding motifs: the conventional *E.coli* one (CTGTN₈ACAG) in *lexA¹-imuA¹-imuB¹-dnaE2¹* cassettes and that observed in the *lexA²-imuA²-imuB²-dnaE2²*

cassette instances of the *Xanthomonadaceae* and the *Pseudomonadaceae* (GTACN₄GTGC). Further evolution in the *Xanthomonadaceae* would have then led to their own *lexA¹* binding sequence [TTAN₆TACTA, (16)], following a deletion of the rest of the *lexA¹-imuA¹-imuB¹-dnaE2¹* cassette. Based on the DnaE1 tree, it seems clear that a major deletion of the *lexA²-imuA²-imuB²-dnaE2²* cassette took place after the split between the *Pseudomonadaceae* and the rest of gamma proteobacteria, since there is no evidence of either the *lexA²* gene or the rest of its accompanying cassette in the *Vibrionaceae*, the *Pasteurellaceae*, the *Alteromonadaceae* or the *Enterobacteriaceae*. A similar deletion must have taken place early in the evolutionary history of the beta proteobacteria, since there is again no evidence of any component of the *lexA²-imuA²-imuB²-dnaE2²* cassette in this bacterial subclass.

Reorganization of duplicated *lexA-imuA-imuB-dnaE2* cassettes in the gamma and beta proteobacteria

The duplication scenario outlined in Figure 4 raises some interesting points. On the one hand, a repeated feature in both gamma and beta proteobacteria concerns the genetic reorganization of the *lexA¹-imuA¹-imuB¹-dnaE2¹* cassette, involving the excision of the *lexA¹* gene from the cassette. This seems to have occurred early in the evolutionary history of the beta proteobacteria, but it must have also taken place independently in the gamma proteobacteria and the *Xanthomonadaceae*. Such a recurring pattern of convergent evolution points to some form of positive pressure towards the split of the four-gene cassette, but it is difficult to speculate on its nature. An appealing possibility concerns the need for more efficient regulation of the constituents of the *imuA¹-imuB¹-dnaE2¹* cassette. Even though operon organization is a straight and effective means of regulation, the inclusion in the operon of its own governing gene (*lexA¹*) reduces the ability to fine-tune the expression of downstream genes. It can be thus speculated that the observed convergent split of the *lexA¹-imuA¹-imuB¹-dnaE2¹* may be due to a requirement to improve repression of the mutagenic *imuA¹-imuB¹-dnaE2¹* cassette in normal conditions, or to boost its expression under adverse circumstances, without influencing negatively on the behavior of the whole LexA regulon. In this context, it is interesting to note a remarkable coincidence. The split of the *lexA¹-imuA¹-imuB¹-dnaE2¹* in the gamma proteobacteria is concurrent with the first instance of a *sulA* gene in this bacterial class (Figure 4). The *sulA* gene (40), encoding a cell-division inhibitor and under mandatory regulation by LexA in *E.coli* and *Salmonella typhimurium*, is absent from all other bacterial classes and phyla. Even though *sulA* is not directly associated with *lexA* in these two species, its first detectable homologs in *Idiomarina loihiensis*, the *Shewanellaceae* or the *Pseudomonadaceae* are in a *lexA-sulA* tandem operon configuration (20) and its transition into the *E.coli* configuration can still be traced through the analysis of its genomic surroundings in the *Vibrionaceae* (data not shown).

Figure 3. Unrooted DnaE2 sequence tree. Shaded areas correspond to established phylogenetic groups. Branch support values below 0.85 or 50% for Bayesian or ML inference phylogenetic analyses, respectively, are not shown. Cassette configurations are shown using constituent genes abbreviations: L—*lexA*, A¹—*imuA¹*, A—*imuA*, B—*imuB*, E—*dnaE2*. P indicates that the cassette is located in a plasmid. Name abbreviations are as in Figure 2. Additional information for each *dnaE2* locus is provided in Supplementary Table S1, which is included as Supplementary Data.

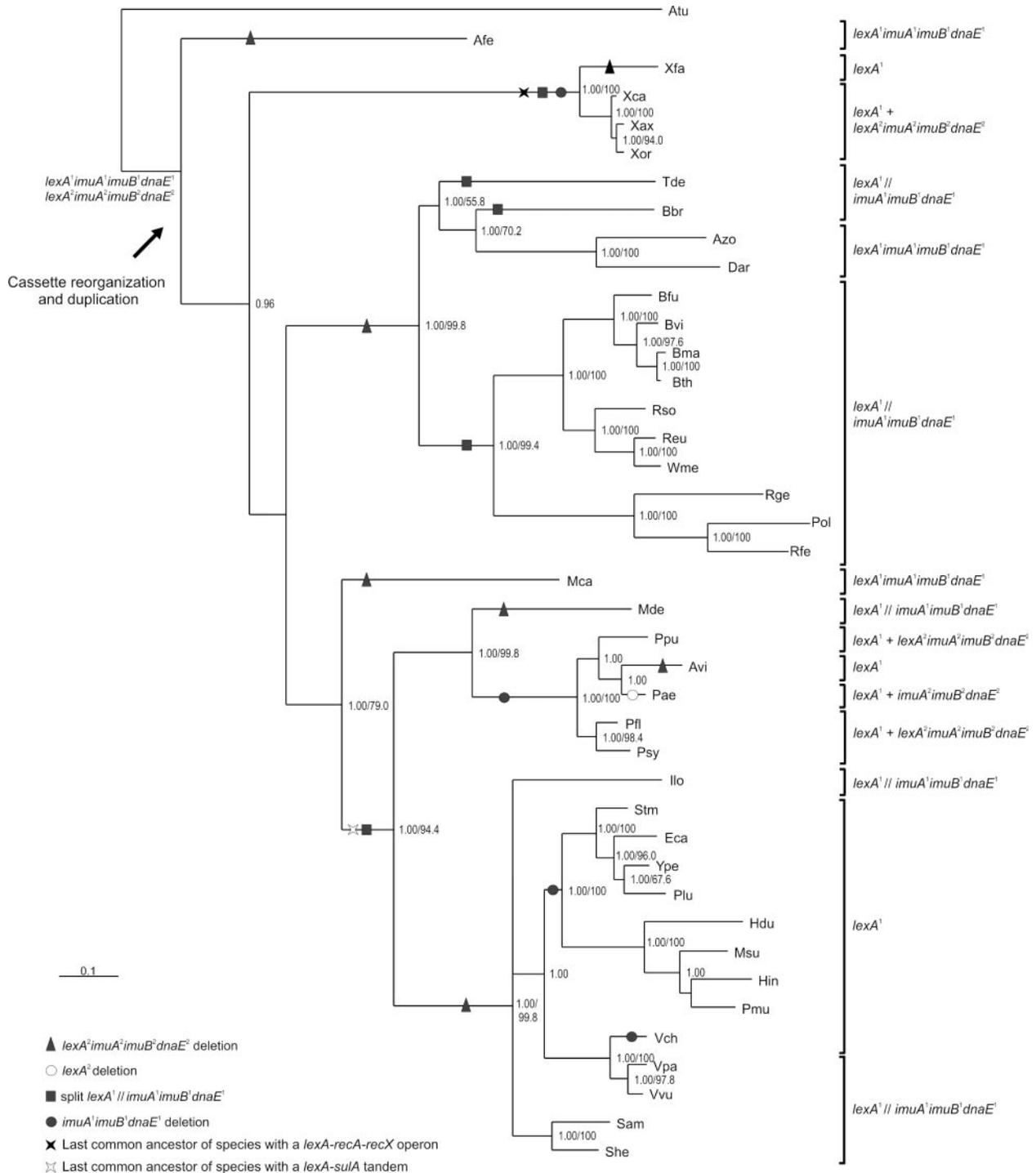


Figure 4. Reconstruction of the proposed duplication hypothesis on a DnaE1 sequence tree of the gamma and beta proteobacteria classes, rooted using *A.tumefaciens* DnaE1 (Atu) as an out-group. Branch support values below 0.85 or 50% for Bayesian or ML inference phylogenetic analyses, respectively, are not shown. Cassette configurations and instances are shown on the right. Deletion and reorganization events are indicated by different symbols (see bottom left of Figure 4 for symbol explanation). Name abbreviations that are not used in Figure 2 are as follows: Eca—*Erwinia carotovora*, Hdu—*Haemophilus ducreyi*, Hin—*Haemophilus influenzae*, Msu—*Mannheimia succiniciproducens*, Plu—*Photobacterium luminescens laumondii*, Pmu—*Pasteurella multocida*, Stm—*Salmonella enterica* serovar Typhimurium, Vvu—*Vibrio vulnificus*, Xfa—*Xylella fastidiosa* and Ype—*Yersinia pestis*. Additional information for each *dnaE1* locus and cassette configurations is provided in Supplementary Table S1, which is included as Supplementary Data.

It is thus reasonable to conjecture that the entrance of *sulA* in the gamma proteobacteria may have played a role in the split of the *lexA¹-imuA¹-imuB¹-dnaE¹* cassette, especially since the *imuA¹* gene shares a degree of homology with *E.coli*

sulA which has been found significant enough to incorrectly annotate this gene as *sulA* in some bacterial genomes (e.g. *P.putida*). This line of reasoning is further supported by what can be observed in the *Xanthomonadaceae*, where the split or

partial deletion of the *lexA*¹-*imuA*¹-*imuB*¹-*dnaE2*¹ gave way to a unique genetic reorganization involving *lexA* and *recA*, another gene sharing residual homology with *imuA*¹, leading to the *lexA*¹-*recA*-*recX* operon observed in this family (16).

Some other questions stemming from this scenario are also of substantial interest. For instance, the complete and final loss of the split *imuA*¹-*imuB*¹-*dnaE2*¹ cassette in the *Enterobacteriaceae* and other families (such as the *Pasteurellaceae*) calls for some explanation, as it appears to have occurred independently. Even though more data are required to shed more light on the matter, it seems relevant to note that the complete loss of the *imuA*¹-*imuB*¹-*dnaE2*¹ is strongly, though not univocally, linked to the emergence and prevalence of an *umuDC* operon in these families (10), which may have offered alternative means for adaptive mutagenesis, thus rendering the *imuA*¹-*imuB*¹-*dnaE2*¹ functionally redundant.

To summarize, the extensive analysis of the evolutionary history of the *imuA*-*imuB*-*dnaE2* undertaken in this work has led to the identification of LGT and duplication events along its evolution, and has substantiated the claim that its mutagenic activity arises from a concerted activity of *imuB* and *dnaE* genes that calls for explicit LexA regulation, in spite of the marked changes in the regulatory sequence of this protein and some drastic turns in the evolutionary history of the *imuA*-*imuB*-*dnaE2* cassette. Nevertheless, the scenarios suggested to explain the evolution of the *imuA*-*imuB*-*dnaE2* cassette do also leave alluring open questions, such as the ultimate origin of the initial actinobacteria *imuA*¹-*imuB*-*dnaE2* cassette, or the influence of two cohabiting *lexA* copies in the evolution of the SOS regulatory network of gamma proteobacteria.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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