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GENE AMPLIFICATION AND GENOMIC PLASTICITY IN PROKARYOTES

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ABSTRACT

Gene amplification is a common feature of the genome of prokaryotic organisms. In this review, we analyze different instances of gene amplification in a variety of prokaryotes, including their mechanisms of generation and biological role. Growing evidence supports the concept that gene amplification be considered not as a mutation but rather as a dynamic genomic state related to the adaptation of bacterial populations to changing environmental conditions or biological interactions. In this context, the potentially amplifiable DNA regions impose a defined dynamic structure on the genome. If such structure has indeed been selected during evolution, it is a particularly challenging hypothesis.

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INTRODUCTION

Gene amplification is a genetic phenomenon that is probably ubiquitous in the genomes of prokaryotic organisms. The foundations for current interpretation of gene amplification were laid mainly through the elegant genetic work of Roth, Hill, and their associates in *Escherichia coli* and in *Salmonella typhimurium* (reviewed in 2, 37, 75, 93), as well as through studies by Rownd and his associates on R-factor "transitioning" in *Proteus mirabilis* (35, 73, 74). These studies allowed the establishment of what we call the canonical model for prokaryotic gene amplification. The quest for unity out of the diversity inherent in biological systems led to analysis of this phenomenon in other prokaryotes. Initially recognized by purely genetic means, the application of molecular biological techniques to study amplification events in prokaryotes has provided deeper insights. This work has expanded the basic tenets and has extended the canonical model. However, because the characteristics of gene amplification in other organisms, with members of the genus *Streptomyces* as a prime example, do not fit so neatly into the canonical scheme, a different paradigm emerges. Studies to define the biological role of gene amplification have provided a wealth of examples to illustrate the phenotypic richness that can be accrued through this phenomenon.

GENOMIC STRUCTURE AND GENE AMPLIFICATION

The Canonical Model

The pioneering studies on gene amplification in *E. coli*, *S. typhimurium*, and *P. mirabilis* were followed by a rapid expansion of knowledge about natural gene amplification in a wide range of prokaryotes. Most of the cases analyzed adhere closely to the basic characteristics of the canonical model. Such examples include *Bacillus subtilis* (34, 43, 67, 102, 108, 111, 113), *Deinococcus radiodurans* (62), *Haemophilus influenzae* (14, 45, 51, 100, 101), *Klebsiella aerogenes* (69), *Pseudomonas aeruginosa* (17), *Rhizobium etli* (27, 89, 90), *Streptococcus faecalis* (109, 110), and *Vibrio cholerae* (31, 65). The central part of Figure 1 (boxed) represents the basic features of the canonical model, as described below.

Most of the early work emphasized the astonishing feature that tandem duplications may affect almost any locus on the bacterial chromosome. However, the frequency of duplication for specific loci varies widely, from 10^{-2} to 10^{-5}. Scanning of the whole *S. typhimurium* chromosome for the occurrence of duplications, employing a single technique, revealed zones that behave as hotspots in regard to tandem duplication, with frequencies as high as 3% (3). Hotspots have been described in every bacterial system analyzed, whether chromosomal or plasmidic. A common feature in every characterized hotspot is the presence

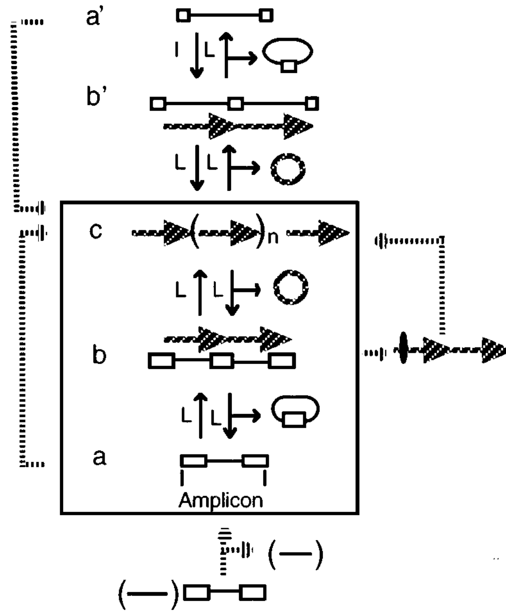

Figure 1 General models for gene amplification in prokaryotes. The central part (*boxed*) illustrates the canonical model; alternate routes are shown outside the box, connected either by solid or broken arrows. Most of the pathways shown here operate sequentially, leading to small increases in copy number. However, routes shown in the left part might operate by bursts of amplification leading to copy number increases of 100 or more. Lower-case letters indicate successive steps in each case. Amplicon structures, *a* and *a*'; tandem duplications, *b* and *b*'; tandem amplification, *c*. Throughout the figure, long repeated sequences are shown as empty rectangles and short repeats by empty squares. Long cross-hatched arrows represent the extent of tandem repeats formed; oval on the cross-hatched arrow (*right*) represents a deletion. (L), legitimate, and (I) illegitimate recombination events. The bottom part shows an amplicon that is "activated" by deleting a large region (*shown in parentheses*) in the vicinity of the amplicon. The resulting amplicon is then amplified in a canonical way (*central part*) or by a sudden burst of amplification (*left part*).

of long repeated sequences (i.e. larger than 1 kb) in direct orientation, flanking the region that undergoes tandem duplication. Structures composed of a DNA segment bracketed by two repeated DNA sequences present in direct orientation were recently dubbed "amplicons" (27, 90), to reflect the fact that they are prone not only to tandem duplication but to amplification as well (see below). The central part of Figure 1 (boxed) schematizes an amplicon structure (*a*), as well as the structures generated during an amplification event. Homologous recombination between the repeated sequences may lead either to a tandem duplication (Figure 1*b*) or to a deletion of the amplicon sequence (not shown).

These structures are thought to be formed by mechanisms entailing unequal exchanges or through circle-excision and reinsertion (see Mechanisms for Gene Amplification). Tandem duplications generally do not lead to loss of gene function (2, 93). Recombination between any pair of long repeated sequences, sharing an adequate level of identity, may possibly foster the formation of tandem duplications. Given the paucity of these sequences in enterobacterial genomes, chromosomal amplicons tend to be large—in some cases encompassing half of the chromosome of *S. typhimurium* (3). Size distribution may become skewed to smaller sizes in some prokaryotes that have a large abundance of repeated sequences, such as *Rhizobium* (25) or *Halobacterium* (94).

The specific nature of the repeated sequences is unimportant in generating a hotspot. Among the repeated sequences that may participate in generating tandem duplications, there are common inhabitants of many genomes, such as ribosomal (*rrn*) operons (3, 37, 38) and insertion sequences (IS) of different kinds (12, 32, 50, 74, 105, 109). Examples of species-specific repeated sequences that participate in amplification are the *r*ecombination *h*otspot *s*equences (*rhs*: Reference 58), found only in *E. coli* (114), or the repeated nitrogenase operons in *Rhizobium etli* (89).

The main factors influencing ability to duplicate at high frequency are the length and sequence conservation between the recombining repeats. Recombination between repeated sequences larger than 300 bp in *E. coli* is strongly dependent on *recA*, especially if those repeats are separated by a proportionally long DNA segment (5). In this regard, null mutations in *recA* provoke a strong decrease in the ability to generate tandem duplications (2, 31, 58, 75, 90, 98, 110), whereas mutants harboring a constitutively activated *recA* allele show an enhanced duplication frequency (20). Small divergences in sequence (10%) between DNA repeats may reduce recombination frequency as much as 40-fold (97). Consequently, mutations in the major editor of genetic recombination in bacteria, the mismatch repair system (86), lead to an increase in tandem duplication mediated by sequence-diverged repeats, such as the *rhs* elements (76). These constraints set an upper limit to the frequency of duplication of a given amplicon.

The role of these constraints is relaxed after a tandem duplication is formed. The large size and perfect homology between the halves of a tandem duplication increase the recombination frequency dramatically. Homologous recombination between the tandemly duplicated sequences may lead either to further amplification (Figure 1*c*) or to deletion, resulting in the reversion to the original genomic structure, at frequencies as high as 150-fold over the frequency of the initial tandem duplication (2, 17, 31, 37, 74, 89, 90, 93). Successive recombination between these members leads to contractions and expansions in length of the tandem array. Thus, formation of amplifications under the canonical

model is a sequential process, where the rate-limiting step is the formation of the initial tandem duplication.

Interestingly, the dynamic nature of DNA amplification continuously generates closed circular structures that consist of monomers or multimers of the amplified unit (Figure 1). These structures will be lost owing to the lack of an origin of replication (27, 38, 58, 100, 109). However, the use of some genetic manipulations may allow such structures to be recovered (27, 58, 82). This process facilitates the molecular characterization of the amplicon sequences.

Extensions of the Canonical Model

The canonical model has been the paradigm for thinking about prokaryotic gene amplification. Its robustness is strengthened by the number of cases, in different prokaryotic species, that can be accurately explained by this model. Moreover, this model can be extended to explain and predict new instances. An interesting extension relates to the apparent stability of some tandem duplications. Theoretical modeling indicates that, in the absence of selection for the duplicated or amplified state, organisms harboring a single copy should become predominant in the population (47). Thus, if a stable tandem duplication is found in a bacterial population, explanations invoking a positive selective value for the duplication or some sort of recombinational avoidance must be sought.

A fascinating example came from the analysis of *H. influenzae* genes involved in capsule production. These genes are found naturally as part of a tandem duplication in most of the isolates (39, 50, 51). "Stability" of the tandem duplication was achieved by deleting one copy of a gene essential for capsulation (50). Reversal of the tandem duplication is frequently accompanied by loss of capsule production (39), which thus explains the predominance of the duplication. According to the canonical model, generation of the tandem duplication is the limiting step for further amplification. Thus, this region should be "activated" for amplification. This prediction has been confirmed (14, 51). Such a situation is schematized in the right part of Figure 1.

A further extension to the canonical model is that duplications may also be formed, albeit at much lower frequencies, by recombination between short repeated sequences (*a*' in the upper part of Figure 1), for example, some tandem duplications in *S. typhimurium*, which can be formed through recombination between the 35-bp long REP elements (98). Once the first duplication is formed (Figure 1*b*), the remaining events that lead to amplification proceed through homologous recombination between the repeats. More extreme examples, generated by illegitimate recombination (23) between shorter sequence repeats (7–15 bp), have been detected spanning the *hisG* (98), the *ampC* (22), or the *lacZ* (104, 107) genes in *E. coli.*

More radical departures from the canonical model are suggested by the extremely high copy number that some amplifications may reach, at frequencies as high as 10^{-6} (12, 22, 44, 104, 107). These amplifications usually comprise small amplicons, and they may establish at copy numbers as high as 100 (Figure 1, *left*). Because it is hard to reconcile such high-level amplification with the sequential steps and mechanisms currently invoked under the canonical model, other mechanisms (78), involving the use of rolling circle intermediates, have been considered.

Note that a particular locus in the genome may form part of different amplicons. Different amplicons will show up depending on the nature of the test used to detect or select the amplified variants. In general, amplicons with long and highly similar direct repeats as borders will amplify at the highest frequency. These are usually large amplicons that give rise to a low number of tandem repeats. When a large number of copies are demanded for a particular selection procedure, smaller amplicons will be detected. In some of these cases, the initial duplication will occur at low frequency, mediated by a *recA* independent, illegitimate recombination.

Amplifications in Streptomyces: A Different Paradigm

Extreme deviations from the canonical model were found in *Streptomycetes*. Initial studies were motivated by the extreme instability, at frequencies usually exceeding 10^{-2}, exhibited by several important traits in these organisms. Among the unstable characters were aerial mycelium production, pigmentation, sporulation, and/or antibiotic production. In some cases, such as in *S. ambofaciens* (55, 56) and *S. lividans* (6), instability is a biphasic phenomenon, where high instability for a certain trait is followed at frequencies as high as 87% by instability for another trait. Frequencies of phenotypic instability can be dramatically augmented by DNA-damaging treatments or by physiological stresses (6).

Genomic analysis of strains obtained from the first phase of instability revealed massive chromosomal deletions, ranging from 250 to over 1500 kb in size (6, 54, 56), with no evidence for amplifications. In strains obtained from the second step of instability, these deletions may extend further, and they are usually accompanied by tandem amplification of specific chromosomal sectors (6, 54, 56), called AUDs (for *A*mplifiable *U*nit of *D*NA). Thus, amplification events in *Streptomyces* are part of a cascade of rearrangements. Available evidence indicates that amplification of the AUDs is either allowed or triggered by large deletions (Figure 1, *bottom*), usually formed on only one side of the AUD (6, 54). After, or concomitant with, the deletion event, there is a burst of amplification affecting the AUD (Figure 1, *left*). Copy number of the amplified regions is extremely high, reaching as much as 500 copies (6, 54). Depending

of the size of the amplified region, this copy number may represent as much as 35% of the total DNA (6, 54). Amplifications in *Streptomyces* stand as the most dramatic example of gene amplification in any prokaryotic organism.

Two types of AUDs are recognized in *Streptomyces*. Type I AUDs usually carry small (<25 bp long) repeated sequences in direct orientation (6, 36, 54, 85); type II AUDs, are flanked by large (0.7–2.2 kb) direct repeats (6, 24, 87). These units bear a superficial resemblance to amplicons that duplicate by illegitimate or legitimate recombination, respectively.

Interestingly, some isolates of *S. lividans* harbor tandemly duplicated copies of a type II AUD, whereas others possess a single copy (21). Isolates carrying a single copy of the AUD amplify at much lower frequencies than do isolates harboring the tandemly duplicated AUD (21). This might be explained assuming, as in the canonical model, that formation of a duplication is the rate-limiting step for amplification. However, derivatives carrying amplifications of an AUD deamplify very slowly (42). Circular structures, consisting of multimers of the AUD, have been observed in some species, such as *S. ambofaciens* (54). Despite the superficial similarities with some structures postulated by the canonical model, the idiosyncratic behavior of amplifications in *Streptomyces* imposes a different paradigm. Specific mechanisms for abrupt gene amplification of the AUDs and the connection with large deletions are considered in a later section.

An unexpected addition to an already alluring model was the recent finding that the chromosomes of many *Streptomyces* species are linear (57, 59, 87). In both *S. ambofaciens* (54) and *S. lividans* (87), AUDs are located near the ends of the chromosome, with deletions frequently removing one end (85). Interestingly, the genome switches from a linear to a circular conformation in strains harboring amplifications (87). These data give clear testimony of the enthralling possibilities for genomic plasticity in prokaryotes.

Genome Structure and Dynamics

The potential for high-frequency DNA amplification in a genome depends to a great extent on the amount, location, and relative orientation of long, repeated sequences. These factors, coupled to the genomic architecture (chromosomal vs plasmidic, linear vs circular, reviewed in Reference 9) contribute to shaping the specific "amplicon structure" of a particular genome.

The interplay between these factors creates some similarities but also striking differences, even between closely related genomes, such as *E. coli* and *S. typhimurium* (Figure 2). The most conspicuous families of long, reiterated DNA elements in *S. typhimurium* are the *rrn* operons and the *Salmonella*-specific insertion sequence IS200. No other IS's have been reported for this organism. A correlation exists between the location of these elements and their use to generate long duplications, some of them overlapping (32: reviewed in

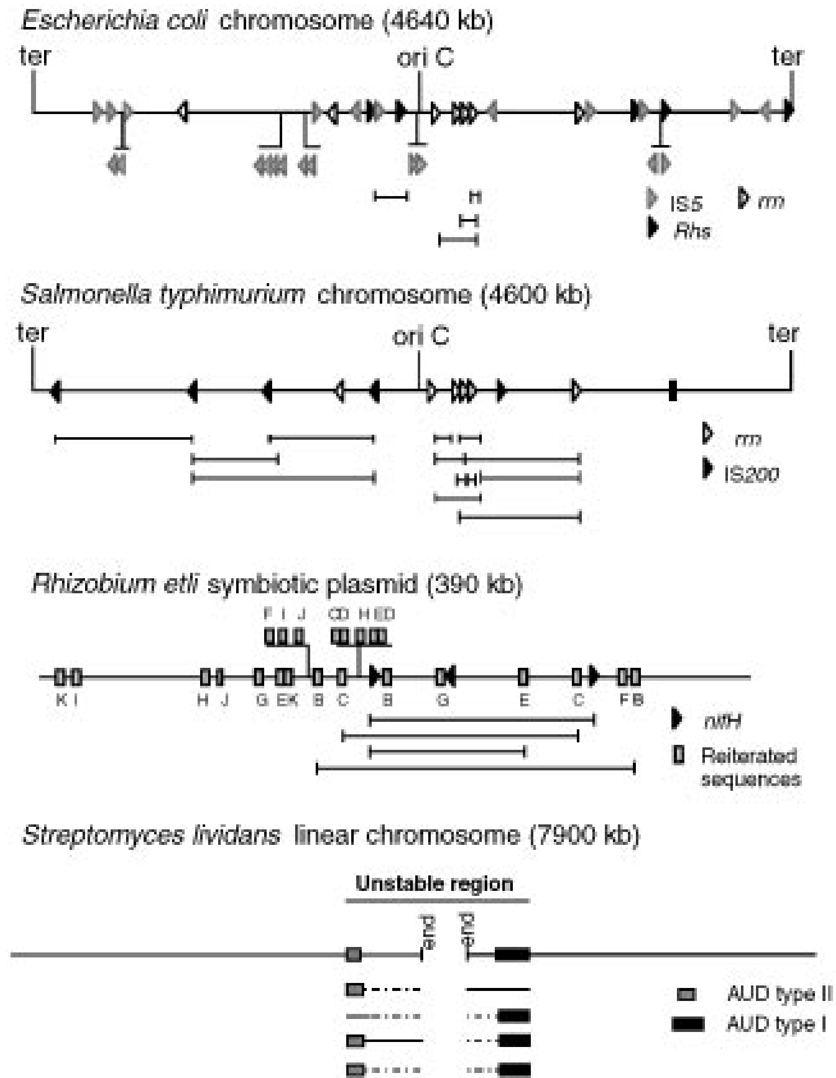

Figure 2 Genomic maps of different bacterial replicons, showing the location of repeated families and corresponding amplicons. *Boxes* and *arrowheads* represent repeated families, coded by shading; *uppercase letters* on the *R. etli* map represent different families. Direction of the arrowheads represent orientation of the respective element. In the maps of *E. coli* W3110, *S. typhimurium* LT2, and *R. etli* CFN42, experimentally demonstrated amplicons are represented by *solid horizontal lines*; *vertical lines* mark the boundary for each amplicon. In the *S. lividans* 66 map, *boxes* on the main map represent the unamplified AUDs. Beneath the map different rearrangements are shown; amplified regions are shown as *boxes*, and regions that are unaffected or deleted are shown as *solid* and *broken lines*, respectively. Information for this figure was collected from References 37, 38, 58, 105 (*E. coli*); 2, 32, 93 (*S. typhimurium*); 30, 89, 90 (*R. etli*); and 57, 85, 87 (*S. lividans*).

93). In fact, most of the genome is subject to frequent duplication events; only the region surrounding the replication terminus is recalcitrant to duplication (93).

Amplicons of similar location and extent between *E. coli* and *S. typhimurium* (Figure 2) are restricted to those involving recombination between the *rrn* operons (37). In *E. coli*, amplicons may be bracketed by specific *rhs* sequences (58). Perhaps the most striking difference relates to the number of IS's. *E. coli* has eight different types of IS's, for a total of around fifty elements (18). In Figure 2 we show the location of the most highly reiterated family, IS5. Specific pairs of IS5 participate in the generation of tandem duplications (105). If most of these elements participate in the formation of tandem duplications, the distribution of amplicons should be different between *E. coli* and *S. typhimurium.*

Highly dynamic genomes have been suggested for organisms that show a distinctively high degree of DNA reiteration, such as *Rhizobium* (8, 26, 25, 91), *Streptomyces* (40), and *Halobacterium* (94, 95).

In *Rhizobium*, the genome is constituted by the chromosome and by large circular plasmids ranging in size from 100 to 1700 kb. Plasmids may comprise up to 40% of the genome (61). In many *Rhizobium* species a single plasmid (the symbiotic plasmid) carries most of the nodulation and nitrogen-fixation genes. *Rhizobium* genomes show a high reiteration, estimated for *R. etli* at 700 repeated elements belonging to 200 distinct families (25). These repeated families are present in the chromosome and in the different plasmids. Amplicons have been found in the plasmids and in the chromosome of *Rhizobium etli* (27, 89, 90, 91) and *Rhizobium tropici* (P Mavingui & R Palacios, unpublished information).

The distribution of repeated sequences as well as the amplicons detected in the symbiotic plasmid of *R. etli* are shown in Figure 2. This plasmid contains 11 reiterated families containing 2–3 elements each (30). One of these families corresponds to the structural genes (*nifH*) for nitrogenase (83). Four amplicons were detected in this plasmid, giving rise to duplications and higher-order amplifications at frequencies ranging from 10^{-3} to 10^{-5} (89, 90). Interestingly, the amplicon bordered by the *nif* structural genes amplifies at the highest frequency. The four amplicons overlap extensively and range from 90 to 135 kb (Figure 2). Partitioning of this plasmid into amplifiable and nonamplifiable zones is evident, since all the amplicons detected are concentrated in a zone of roughly one third of the plasmid, covering most of the symbiotic genes (90). A similar case of partitioning was seen for the R-factor NR1 in *Proteus mirabilis*; this plasmid can be separated into amplifiable (the R-det) and nonamplifiable (RTF) regions (35, 73, 74).

Regions subject to deletion and amplification events in *Streptomyces* are located close to the ends of the linear chromosome (Figure 2). Most of the deletion events remove one end, although some remove both ends. Likewise,

amplification events frequently act on one AUD (85, 87). The reasons for this particular arrangement are unknown. The dispensability of the unstable region for growth under laboratory conditions was initially rationalized by assuming that the unstable region contains relatively few genes. However, the existence of profound changes in patterns of protein synthesis in *S. ambofaciens* strains harboring extensive deletions indicates that these regions are actively expressed (16). A second alternative is that chromosome ends might play a role, as yet undescribed, on the generation of amplifications and deletions. In that regard, it should be interesting to study the generation of amplifications in other bacterial species that contain linear chromosomes, such as *Borrelia burgdorferi* (10) and *Agrobacterium tumefaciens* (1).

The genomes of some archaebacteria also contain a large number of reiterated DNA sequences and undergo frequent rearrangements (11, 94, 95). The genome of *Halobacterium salinarium* harbors an estimated 500 repeated elements, belonging to as many as 50 different families (94). Most of this repeated DNA consist of IS elements (11). Two genomic compartments of differing GC-content (FI and FII) have been found in the genome of *H. salinarium* (79). The FII fraction is constituted by a large plasmid and by several long stretches (called "islands" in *H. salinarium*) of relatively AT-rich DNA embedded into GC-rich chromosomal sequences. The IS elements are concentrated on the FII islands (79). Many rearrangements observed in this species are concentrated in the islands or are due to the movement of the IS to the FI fraction (33, 80, 81). Islands, also called "oases," have been found also in *Haloferax volcanii* (13). Although few examples of gene amplification have been described in haloarcheal species (92), it is expected that localization of the islands will play a role in defining the amplicon organization in these species.

MECHANISMS FOR GENE AMPLIFICATION

Figure 3 shows the different mechanisms proposed to explain the formation of gene amplification in prokaryotes. Two of these, the unequal crossover (UC) and the circle-excision and reinsertion (CER) mechanisms, might explain amplification under the canonical model (2, 37, 75, 93). The rolling circle (RC) mechanism was proposed to explain instances of abrupt gene amplification, as seen in *Streptomyces* (112) or in some "induced" amplifications in *B. subtilis* (78).

The UC and the CER mechanisms involve normal rounds of DNA replication; the UC mechanism in the form of a replication bubble, whereas the CER mechanism requires the existence of two replicons in the cell. Alternatively, the CER mechanism may use a replication bubble as an intermediate, excising a circle from an arm of the bubble and reinserting it into the other arm. In the UC

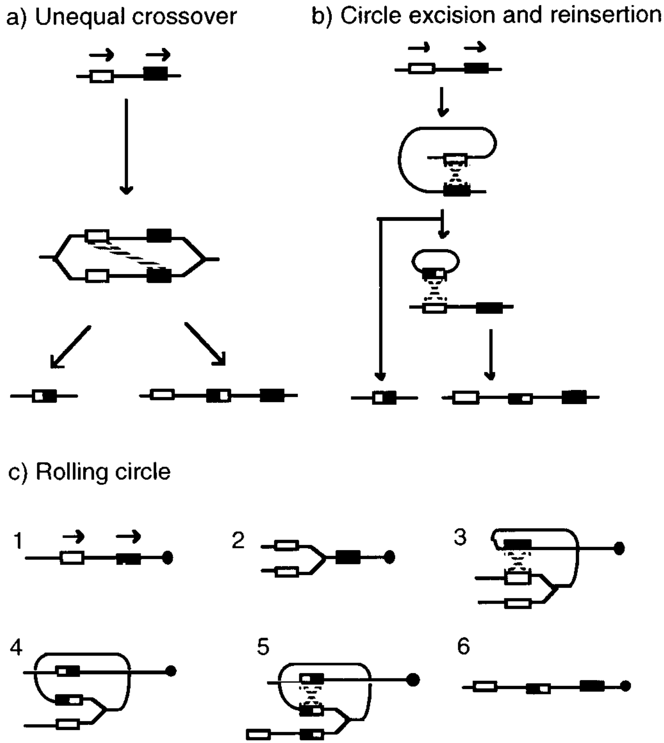

Figure 3 Mechanisms for gene amplification in prokaryotes. Repeated sequences are represented by *boxes of different shading*; *boxes with mixed shading* represent the join points. Recombination events are represented as *broken lines* (see text).

mechanism, a reciprocal intermolecular recombination generates two products (a deletion and a duplication); a second, intramolecular recombination is needed to resolve the resulting dimeric molecule (Figure 3). Alternative proposals that recombination might proceed by half-reciprocal events would generate a full product (a deletion or a duplication) and a broken molecule (60, 96). The rate-limiting step is the initial recombination; the second recombination event apparently occurs as the site-specific recombination event usually involved in chromosome (7) or plasmid partitioning (15).

In the CER mechanism, a reciprocal intramolecular recombination generates a deletion product and an excised circle; this circle then recombines with a second molecule to produce the duplicated product. The efficiency of circle integration may be very high (60); this can be explained by the extensive homology between the circle and the recipient molecule, or by incorporating the

possibility of half-reciprocal events. A half-reciprocal recombination would generate a closed deletion product and a broken circle. Based on genetic evidence, Mahan & Roth (60) proposed that these linear molecules can be loaded by the RecBC enzyme; this promotes circularization, generating a circle now activated for integration, owing to the presence of RecBC.

The operation of the CER mechanism is made plausible by the demonstration of circular intermediates in different systems (27, 38, 58, 100, 109). Studies on the resolution of a nontandem duplication during spore formation in *B. subtilis* failed to provide evidence for the UC mechanism, suggesting instead the CER mechanism (102). However, studies of resolution of a tandem duplication in a plasmid system in *E. coli* show that all the products in wild-type cells carried a dimeric plasmid, containing a deletion and further amplification of the original tandem duplication (19), supporting the UC mechanism. More studies are needed to ascertain if there are species- or substrate-specificities for operation of these mechanisms.

Operation of the UC or CER mechanisms would lead to gradual increases in copy number, requiring several generations to reach high-level amplification. Thus, these models are inadequate to explain sudden increases in copy number, as in *Streptomyces*. The RC mechanism (112) accounts nicely for the idiosyncrasies of the *Streptomyces* model. This model proposes the entrapment of a replication fork into a circular structure that stays attached to a chromosomal arm. This structure would be generated by intramolecular recombination between repeats in direct orientation, while the replication fork is traversing the AUD (Figure 3). A fork captured in such way would behave like a rolling circle, leading to the generation of tandem amplifications to a very high copy number. Resolution of this structure would be achieved by recombination between the original repeats in the sister arm, generating an amplification without a deletion (Figure 3). Another alternative is by recombination downstream of the site of the original recombination, generating an amplification accompanied by a deletion. This proposal is flexible enough to accommodate the possibility of large deletions prior to amplification, as seen in species that present biphasic instability. These would occur through recombination, without trapping of a replication fork in the AUD structure. It is unclear if the ends of these linear chromosomes play a direct role in illegitimate recombination.

Operation of the RC mechanism was tested in *Bacillus subtilis* (77, 78) by chromosomal integration of an activable, rolling circle–type origin in the vicinity of an artificial amplicon structure. Short-term activation of replication from this origin provoked amplification to a very high copy number, revealing at the same time unstable intermediates predicted by the RC mechanism (77, 78). However, prolonged activation of replication leads to deletion of the array, probably due to enhanced recombination provoked by the generation of single-stranded DNA (71). Enhanced recombination was only seen with a rolling

circle–type origin in *B. subtilis* (66); however, recent results obtained with a similar system in *Rhizobium etli* indicate that enhanced recombination may occur with a theta-type origin as well (E Valencia & D Romero, unpublished results).

Activation of a replication origin lying nearby might be involved in some of the instances of sudden, high-level amplification observed previously. For instance, high-level amplification of the *argF* gene in *E. coli* occurs at high frequency only in Hfr strains that transfer early the *argF* marker (12, 44). Conceivably, replication primed from the origin of conjugal transfer would generate a rolling circle–type structure, similar to the one seen in Figure 3. High-level amplification of the *lacZ* gene in *E. coli* (104, 107) may well depend on a conjugal transfer origin, since amplification was observed in strains harboring *lacZ* on an F-prime plasmid. Similarly, tandem amplifications in *Bacillus subtilis* were initially reported as exceedingly unstable, employing an experimental approach that included an "artificial" amplicon (111). Elevated instability was later shown to be dependent on the remnant of a rolling circle–type origin and its activating protein, inadvertently introduced during the construction of the artificial amplicon (113).

BIOLOGICAL ROLE OF GENE AMPLIFICATION

The life of bacteria has been colorfully described as "an adaptation to a feast or famine existence" (46). Genetic regulatory mechanisms in bacteria are normally sufficient to cope with these demands. However, adaptation to new, extreme conditions or episodic situations may impose severe demands on the ability of regulatory systems. It has been argued that the biological role of gene amplification in prokaryotes might be related to adaptation to these conditions (2, 88, 91, 93). According to this hypothesis, overexpression of gene products through gene amplification may confer the phenotypic advantages needed for survival. The amplified state would remain as long as the selective condition exists; when the selective condition disappears, haploid segregants would dominate the bacterial population. Different examples of natural gene amplification support this proposal.

Several instances refer to increased resistance to antibiotics. A well-known case is the amplification of the resistance determinant of plasmid NR1 in *Proteus vulgaris* (35, 73, 74). Amplification of the R-det region ("transitioning") was seen upon growth of *P. mirabilis* cells harboring NR1 in medium containing an antibiotic to which resistance is conferred by the R-det. Increased antibiotic resistance correlates well with the degree of amplification. Amplification was mediated by recombination between IS1 elements flanking the R-det. Deamplification, accompanied by a decrease in antibiotic resistance, was seen upon culturing in antibiotic-free medium (35, 73, 74).

Similarly, amplification of plasmid genes confers increased resistance to tetracyclin in *Streptococcus faecalis* (109, 110). Amplification of specific chromosomal regions is also responsible for enhanced resistance in *Escherichia coli* to ampicillin (22) or sulfonamides (70), and for increased resistance to methicillin in *Staphylococcus aureus* (63). In every case, deamplification leads to diminished antibiotic resistance.

Gene amplification might be involved in adaptation to other environmental stresses, such as resistance to heavy metals. *Thiobacillus ferrooxidans* plays an important role in the biogeochemical cycles of iron and sulfur. A recent report suggests that experimental adaptation for resistance to toxic metals (As and Zn) might be gained through amplification of chromosomal regions (48).

Adaptations for growth under conditions of nutrient scarcity can also be achieved through gene amplification. Chemostat selection experiments demonstrate that tandem chromosomal duplications allow improved growth of *Salmonella typhimurium* under limiting concentrations of malate (99, 103), arabinose, melibiose, or sorbitol (99). Improved permeation of the limiting substrate is partially responsible for this effect (99). Similar experiments in *E. coli* show that tandem duplications permit faster growth under limiting lactose concentrations, apparently due to increased synthesis of β-galactosidase (41, 104).

Growth on several "exotic" carbon sources can also be stimulated by gene amplification. In this regard, growth of *Klebsiella aerogenes* in xylitol, an unnatural carbohydrate, is dependent on tandem amplification of the ribitol dehydrogenase gene region. Ribitol dehydrogenase has a very low affinity for xylitol; thus, enzyme overproduction allows growth on this carbon source (69, 88). Bacteria belonging to the genera *Alcaligenes* and *Pseudomonas* possess surprising catabolic activities toward several pollutants, including alkanes and halogenated compounds, such as 3-chlorobenzoate (3-Cba). Prolonged culturing of *Alcaligenes eutrophus* (28, 29, 72) or *Pseudomonas* sp. B13 (84) cells on media containing 3-Cba selects for variants carrying tandem amplification of the plasmid-encoded genes for 3-Cba-degrading enzymes. Tandem gene amplification has also been implicated in the phenotypic reversal of a mutation blocking alkane utilization in *Pseudomonas putida* (64).

Perhaps the best examples of the role of gene amplification in adaptive processes came from studies of pathogenic or symbiotic interactions. Many isolates of *Vibrio cholerae*, including epidemic strains, harbor a tandem duplication of the cholera toxin gene region (*ctx*). Interestingly, it was recently shown that *ctx* is part of a filamentous phage that generates the tandem duplication of *ctx* upon lysogenic conversion of *V. cholerae* (106). Further amplification of the *ctx* region has been observed during the course of a mouse infection, correlating with increased virulence (31, 65). Natural derivatives carrying amplifications have been isolated from humans (4). In a related example, many invasive strains of

Haemophilus influenzae carry a "stable" tandem duplication of genes involved in capsule formation (39, 50, 51). One third of the *H. influenzae* strains isolated from blood or cerebrospinal fluid of children with meningitis carry further amplification of the capsulation region (14). Increased capsulation afforded by gene amplification is thought to be an important factor for invasiveness (14, 51, 68). Another related example is associated with transition to mucoidy in *Pseudomonas aeruginosa*. In the course of infections by *P. aeruginosa*, bacterial populations are frequently overtaken by mucoid, alginate-overproducer derivatives. These derivatives are one of the main causes of persistent infections. Although the basis for this transition is multifactorial, it has been demonstrated that a chromosomal tandem amplification is implicated in this transition (17).

The benefits of gene amplification might be extended to other organisms, as seen in symbiotic interactions. For instance, in certain genetic backgrounds, tandem duplication of a region in the symbiotic plasmid of *Rhizobium etli*, the symbiont of the bean plant, promotes increased nitrogen fixation in this host (D Romero, unpublished data). Similarly, amplification of a region in the symbiotic plasmid of *Rhizobium tropici* leads to enhanced production of nodulation factors, which are necessary for nodule formation (P Mavingui & R Palacios, unpublished results). A further example is seen during the mutualistic interaction of aphids with bacterial endosymbionts of the genus *Buchnera*. The main function of the endosymbiont is the supply of amino acids, specially tryptophan, for the aphid. Genes encoding anthranilate synthase (*trpEG*) in *Buchnera aphidicola* are located in a plasmid, which is arranged as tandem repeats of four to ten copies of *trpEG* (52). Thus, amplification of *trpEG* would provide the means to overproduce tryptophan for the aphid host. This plasmid-encoded arrangement is prevalent in *Buchnera* species from aphids with a high demand for tryptophan. Alternative arrangements are seen in endosymbionts from aphids with conceivably low demand for tryptophan. These include single chromosomal copies of *trpEG* or a plasmidic arrangement with a single active copy of *trpEG* and six pseudogenes in tandem (53).

CONCLUSIONS AND PERSPECTIVES

Gene amplification events provide several opportunities for the bacterium. First, tandem amplification endows a subset of the bacterial population with the capability for overexpression of defined genomic regions. This capability might be relevant for short-term adaptation. Second, tandem amplification is a transient characteristic, which may return to baseline conditions without altering the structure of the genome. Third, gene amplification provides not only immediate quantitative changes, but also new substrates for evolution. Amplified genes can receive different mutations, thus allowing the evolution of new functions, while leaving the original copies unscathed. These opportunities allow

the bacterial genome to become truly experimental. In the short term, if a particular gene amplification makes the bacterium best fitted for a particular environmental niche, whether episodic or entirely new, it will be strongly selected for. If not, there are two possible outcomes: Either the amplified region reverts to the original state or the amplified copies may diverge by mutation. These opportunities might be particularly relevant for prokaryotic organisms, whose mode of reproduction is largely clonal.

A central biological question is whether the amplicon structure of a genome is subjected to selection during evolution. No direct evidence exists at present to confirm or rule out such a hypothesis: Arguments can be made both in favor or against it. In many cases a defined amplicon clearly confers a selectable advantage to the organism. On the other hand, the amplicon structure of several genomes is strongly influenced by the position of mobile reiterated elements and is thus continuously changing. Presumably, some amplicons have been positively selected during evolution whereas others are present as a collateral result of evolutionary forces centered outside the amplicon itself.

Genome sequencing projects (see Reference 49 for a review) will certainly provide new insights into the amplicon structure of the genome and its evolution. Analysis of the DNA sequence of a complete genome allows the prediction of potential amplicons contained in it, as well as their coding characteristics. Hypotheses on their biological role may then be derived and analyzed with the tools of modern molecular biology. Comparative genomics will help to define the evolutionary history of genomes in terms of their amplicon structure.

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