Natural genetic engineering, adaptive mutation & bacterial evolution

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ABSTRACT

Molecular genetics has taught us new ways to think about bacterial genomes and how they change during evolution. We have elucidated the structure of genetic determinants that range from protein coding sequences and 5’ regulatory regions to operons, regulons and whole chromosomes. At all levels we find hierarchically organized systems composed of shared domains and motifs rather than units. This composite organization implies an evolutionary role for natural genetic engineering systems to combine and rearrange genomic components. Appropriately, molecular geneticists have also discovered a wide range of biochemical complexes in bacterial cells for reorganizing DNA molecules in many different ways. Some natural genetic engineering systems work on single bases, some on sequences sharing limited or extensive homologies, and some on large genomic segments without any requirement for sequence homology in DNA joining. The real-world ecological importance of such genome reorganization capabilities can be seen in the incorporation of similar regulatory elements into different regulons involved in diverse aspects of bacterial physiology and behavior, in the dissemination of multiple antibiotic resistance and complex pathogencity determinants, and in the evolution of catabolic functions for xenobiotic compounds. The phenomenon of adaptive mutation demonstrates that the action of these natural genetic engineering systems can be regulated by cellular signal transduction pathways. Thus, important aspects of genetic change can be linked to biological response systems during bacterial evolution.

INTRODUCTION

Molecular genetics has taught us many lessons about the "driving forces" in microbial ecology and microbial evolution. Among the most important of these have been lessons about how bacterial genomes are organized as hierarchies of modular components, how bacterial genomes undergo structural change through the action of natural genetic engineering systems, and how natural genetic engineering systems are subject to regulation by cellular control circuits. Moreover, bacterial systems such as antibiotic resistance, xenobiotic degradation, and new pathogenicity combinations provide our most fully documented examples of evolution in action. Thus, prokaryotic molecular genetics provides a dynamic vision of the evolutionary process based on cellular functions rather than localized random genetic change (2, 87, 90).

MODULARITY OF BACTERIAL GENOMES: Hierarchically organized systems rather than units

Beginning in the 1950s with the work of Monod and Benzer, molecular genetics has relentlessly deconstructed individual "genes" into their component parts and revealed the architecture of multilocus genetic determinants underlying the expression of particular phenotypes. At all levels, the genome is composed of nested systems rather than units (Table 1). A few examples will clarify this "systems view" of the genome.

We used to think of proteins as unitary structures with each part of the polypeptide chain integrated with the rest. Thus, the open reading frame (ORF) encoding that protein was conceptually a single unit of function and evolution. Today, however, we realize that proteins are comprised of separate domains with distinct and separable functions. The alpha and omega segments of *E. coli* beta-galactosidase (104) and the DNA-binding and cooperativity regions of lambda cI repressor (72) were recognized early as examples of separable domains. With extensive sequencing, we are able to recognize the signatures of various functional domains, such as ATP- or DNA-binding motifs, and assign proteins to families which share certain domains in common, such as the histidine kinases and response

regulators of 2-component control systems (96). The fact that domains can be swapped *in vitro* and retain their functionality shows that they are separable components of the entire protein, whose activity reflects the interactions of its different domains.

The operon theory introduced a new kind of genetic element, the *cis*-acting regulatory site (77). Today we call these elements binding sites for transcription factors or, more generally, for any protein that recognizes a specific oligonucleotide motif (as in replication, recombination or DNA condensation). These binding sites are required both for expression (e.g. promoters) and for proper regulation. For many binding sites, we recognize subcomponents, such as the -10, -35 and spacer regions of canonical σ70 promoters (31). Thus, the functional entity of regulated gene expression is not a unit but rather a composite of 5' and 3' sites and ORF sequence(s) encoding the multidomain protein(s) of a particular operon (77, 90). Similarly, other kinds of genetic determinants, such as origins of replication or site-specific recombination substrates display composite organizations composed of multiple recognition sequences (17).

Most transcription factor binding sites are repetitive elements found at multiple locations in the genome. Promoters specific for various sigma factors and recognition sequences for multilocus control proteins such as Crp (80) and LexA (107), are examples of repeated motifs which integrate different operons into higher order regulatory networks. In ways that we are now trying to explain, these regulatory connections between different genetic loci define much of the control circuitry that allows bacteria to maintain homeostasis during growth and undergo various cellular differentiations when growth ceases (36, 46, 54, 59). In some cases, regulatory hierarchies are multicellular and not restricted to individual cells. One of the most exciting recent developments in microbial genetics has been the realization that intercellular signalling and quorum-sensing systems have been integrated into the regulatory regimes controlling many distinct bacterial phenotypes (27, 32, 53, 69, 81, 98). In the Gram-negative bacteria, the signals are generally acyl homoserine lactones (AHLs) that are synthesized by enzymes encoded by *luxI* homologues and detected by

transcription factors encoded by *luxR* homologues. The incorporation of the *luxI* and *luxR* homologues needed for AHL signalling into distinct functional systems is yet another illustration of genomic modularity. Examining the different ways these determinants are organized in each regulon, it appears that the evolutionary process frequently rearranged the orientation and spacing of the *luxI* and *luxR* homologues (Figure 1; 81).

The modular and hierarchical organization of bacterial genomes has major implications for the fundamental processes of change during evolution. In particular, cellular capacities for rearranging and stitching together different components in a manner analogous to our own genetic engineering are needed to change the functional architecture of a genome (86, 87). How common are such functions in bacteria?

NATURAL GENETIC ENGINEERING SYSTEMS

The basic operations of classical bacterial genetics involve intercellular transfer of genetic information and recombination between DNA molecules, both homology-dependent and homology-independent. There have long been many indications of the cellular capacities for joining DNA sequences: chromosomal integration of temperate phages and sex factors, cloning of chromosomal markers into episomes by specialized transduction and sex factor excision, formation of deletions, duplications and genetic fusions, regulation of gene expression by DNA rearrangements, and the movements of transposable elements within and between genomes (4, 83, 84, 89). Although most of these events used to be treated as “illegitimate recombination,” we now know that they are regular and adaptively important features of genome flexibility in bacteria. Such flexibility is needed to survive in environments that may change rapidly in predictable or unpredictable ways.

The phrase “natural genetic engineering” is meant to encompass all those biochemical systems which bacteria and other cells use to change the information content of DNA molecules. These systems range from activities which carry out localized mutagenesis to those which generate wholesale rearrangements of major genomic components (Table 2).

Natural genetic engineering systems are frequently made up of many components, and there is no correlation between the nature of the genetic change and the biochemical complexity of the responsible proteins. For example, the -1 frameshift that reverses the effect of the *lac33* mutation in response to stress is a single point mutation, but the frameshifting event depends upon the many sequentially acting components of the RecABC pathway (24, 44, 45, 55). It is interesting to note that the recently sequenced genome of a cyanobacterium contains 99 ORFs with transposase homologies (51), and the genome of *B. subtilis* contains at least ten complete or partial prophages (56).

There are three important points to make about the natural genetic engineering systems listed in Table 2:

(a) They mediate a wide range of DNA changes, from base substitutions and frameshifts through deletions, inversions, duplications, fusions and transpositions.

(b) Many of the systems work independently of genetic homology. Topoisomerases and the unknown activities which mediate *in vivo* deletions and duplications generally prefer short sequence homologies (1, 109), but they can join completely non-homologous sequences (10, 13, 49). The action of transposable elements depends on ligating free 3' hydroxyl ends of the donor to 5' phosphate ends of the target (14, 15, 43, 67, 82). This means that target specificity is independent of the ability of donor and target sequences to form a heteroduplex and is determined by the binding affinities of the cognate transposase and accessory proteins.

(c) Multiple genetic changes mediated by natural genetic engineering systems often do not occur independently of each other. For example, *N. gonorrhoeae* uses homologous recombination between silent *pilS* cassettes and the expressed *pilE* locus to generate pilus antigenic variation, and multiple base substitutions result from a single recombination event (28, 97). Transposable elements can be activated to go through multiple recombination events and accumulate at distinct places in the genome. Most transposable elements duplicate as they mediate DNA rearrangements, either because replication is

inherent to the rearrangement process (82) or, in cut and paste events, because the donor site is repaired by recombination with a homologue carrying a copy of the inserted element (14, 40). Thus, related transposition or rearrangement events can occur at multiple genomic locations. It is possible that such coincident events account for the “bursts” of increase in IS element copies observed in dormant cultures of *E. coli* (68).

NATURAL GENETIC ENGINEERING SYSTEMS AT WORK IN REGULATION AND EVOLUTION

The idea of natural genetic engineering systems playing a major role outside the laboratory is no longer new to microbiologists. We are accustomed to thinking about the roles of plasmids, phages, transposons and integrons in the evolution of antibiotic resistance determinants that cause real-world problems in medicine and agriculture (e.g. 42, 74). There are many additional examples we have of ecologically important phenotypes where DNA rearrangements play a role in ongoing regulation or particpated in the formation of the underlying genetic structures. Looking at pathogenicity and xenobiotic degradation functions provides illustrative examples:

• Antigenic variation of gonococcal pili by gene conversion (97). We can infer that this process occurs in nature because of the genetic variability observed in infections, where mixtures of pilus types are recovered and, in experimental infections, where recovered pilus types do not correspond to the infecting type (28). *N. gonorrhoeae* also controls the synthesis of other surface proteins by Rec-independent unequal crossing-over or replication slippage between pentanucleotide repeats in the coding sequence (97). The occurence of similar kinds of repeats in adhesin coding sequences in the *Helicobacter pylori*, *Mycobacterium genitalium* and *Mycobacterium pneumoniae* genomes has led to the proposal that gene conversion and Rec-independent unequal crossing-over or replication slippage also play roles in antigenic variation in these species (26, 47, 100).

• Multiphasic antigenic variation in *Borrelia hermsii* and *Borrelia burgdorferi* by recombination between silent, promoterless *vmp* and *vls* sequences and telomeric

expression sites on linear minichromosomes (3, 26, 114). The Vmp and Vls lipoproteins are the major antigenic determinants on the bacterial surface. Traces of these events can also be found by PCR analysis of field isolates (48). In addition, it appears that *Borrelia* spp. use post-recombination gene conversion events to further diversify their antigenic repertoire (25, 76).

• Evolution of autotransporting (AT) virulence determinants in Gram-negative bacteria by fusion of an AT domain to a variety of virulence factors (60).

• Investigation of the genetic basis of bacterial pathogenicity has indicated that different strains became pathogens by the acquisition of episomes carrying determinants for different kinds of virulence factors. These episomes include phages (35, 106), plasmids (64, 71) and extended chromosomal insertions termed “pathogenicity islands” (PAIs, 38). An examination of the boundaries of these PAIs (Figure 2; 34, 38) clearly indicates that most of them arrived at their chromosomal sites by known natural genetic engineering mechanisms, either site-specific recombination similar to temperate phage integration (UPEC 536 and J96 and *V. cholera*), by the mediation of IS elements (*Y. pestis*) or by one of these two mechanisms, both of which generate flanking oligonucleotide direct repeats (*D. nodosus* and *H. pylori*). The choice of stable RNA loci for integration (EPEC, UPEC, *S. enterica* and *Y. enterocolitica*) is also reminiscent of temperate phage integration (12). Comparing the Cag PAIs of some strains of *Helicobacter pylori* also reveals further action of IS elements (100).

• In recent years, concerns about enhancing bioremediation activities have directed attention to xenobiotic degradation loci in soil bacteria. Patterns of modular rearrangements have been observed in combining “upper” and “lower” pathway determinants to endow single species with the capacity to mineralize aromatic hydrocarbons (110). Many of the catabolic determinants for complete or partial degradation pathways are carried on transposons or are flanked by IS elements (75, 103, 112). Selection for simple modification of a haloaromatic degradation pathway often results in extensive DNA

rearrangements (50), and comparison of toluene and chlorotoluene degradation operons reveals the same kinds of coding sequence rearrangements described above for *luxR* and *luxI* homologues (103).

ADAPTIVE MUTATION, ENVIRONMENTAL INPUTS AND THE EPISODIC NATURE OF NATURAL GENETIC ENGINEERING

It has been known for several decades that many kinds of environmental factors have dramatic influences on mutation frequencies in bacterial cultures. Generally, as in the case of the SOS response (107), these mutagenic influences have been seen as byproducts of repair processes. However, knowledge about natural genetic engineering systems now makes it possible to see DNA change as a useful cellular response to stress, frequently called "adaptive mutation" (19, 78, 90). Three cases of DNA change regulated by culture conditions have been well documented.

(1) In *Alcaligenes eutrophus* strains, an extremely high frequency of both plasmid and chromosomal mutations has been found in the survivors of incubation at 37 C on medium containing methionine, cysteine or serine (102). Up to 80% of the survivors carry mutations affecting many phenotypes (Lys^-, Thr^-, loss of autotrophy, pigment excretion, inability to utilize nitrate or ammonia as nitrogen sources, sensitivity to heavy metals, etc.), and many mutants carry multiple lesions (65). Many of the "survivors" retain thermosensitivity on rich medium, indicating that their ability to grow on the selection medium was not the result of a stable mutation. Some phenotypes, such as lysine or threonine auxotrophy, appear to be specific to "temperature-induced mutagenesis and mortality" (TIMM) because they cannot be recovered by penicillin selection after growth at 30 C. Examination of plasmids carrying TIMM-induced mutations reveals IS element insertion and excision events as well as extensive rearrangements which may be IS-related (99). It appears that *A. eutrophus* strains possess two traits that can lead to rapid environmentally-triggered genome modification: (1) the ability of some cells to enter a transient state in which they can proliferate at 37 C on enriched medium, and (2) a control system for derepressing IS

element activity at 37 C. This fascinating example of regulatable hypermutation apparently involving IS elements merits further attention.

(2) The best-known case of adaptive mutation is reversion of the *lac33* frameshift in a *lacI-lacZ* fusion on an F'*lac* plasmid (9). A particular class of -1 frameshift reversion events occurs at high frequencies on selective medium but not during normal growth (22, 79). Detailed studies have shown that Rec functions (24, 44, 45), F *tra* functions (23, 29, 73) and Dna Polymerase II (21) are all required for adaptive frameshifts to occur. Thus, the underlying events involve a complex set of protein factors. Among these, the F *tra* functions are known to be regulated by the host Arc (*a*erobic *r*espiration *c*ontrol) function (92) and activated by the conditions prevailing during selection: dense, highly aerobic cells unable to proliferate (70). Moreover, additional plasmid and chromosomal mutations are stimulated by the Lac selection and are found among the *lac33* pseudorevertants at frequencies which indicate that they did not arise in independent events (20, 101). Thus, the Lac selection appears to induce a hypermutable state of the kind postulated by Hall (41). In other words, the underlying processes creating these additional unselected mutations must be activated by aerobic starvation.

Aerobic starvation is also known to be responsible for activating the DNA rearrangements in the first clearly documented adaptive mutation system, the formation of *araB-lacZ* coding sequences fusions (61, 66, 85). This is one example of the general Casadaban method developed for fusing any N-terminal coding region to *lacZ* (11). The Casadaban method serves as an experimental model for the creation *in vivo* of multi-domain coding sequences. In Casadaban's original conception, the transposable Mu prophage served simply as mobile homology for inserting a decapitated *lacZ* into many genomic locations (Figure 3), but our work showed that Mu sequences and transposition functions play an active role in fusion formation (Figure 4; 62, 90, 91). The remarkable initial observation was that *araB-lacZ* fusions never occured during normal bacterial growth ($<10^{-10}$ per CFU) but could be recovered at high frequencies ($\sim10^{-5}$ per CFU) after a few

weeks incubation on selection plates or following prolonged starvation (61, 85). Studies of the genetic requirements for fusion formation have identified a number of essential cellular regulatory functions: IHF and HU (91), ClpPX protease (88; JAS & G. Maenhaut-Michel, unpublished), RpoS (30), and Crp (JAS & G. Maenhaut-Michel, unpublished). Taking advantage of Mu genetics and the thermosensitive Mu*cts62* repressor in this system, it has been established that the triggering of fusion formation by starvation includes two experimentally separable effects: (1) prophage derepression in stationary phase to permit Mu A transposase expression, and (2) activation of strand transfer and subsequent DNA processing steps to complete fusion formation (Figure 4; 62; JAS et al, unpublished). Studies of Mu*cts62* derepression using a pE-lacZ reporter system (105) have shown that ClpPX and RpoS but not Crp play a role in stationary phase derepression (C.Ranquet, S. Lamrani, M.-J. Gama, G. Maenhaut-Michel, A. Toussaint and JAS, unpublished). Thus, it may be hypothesized that starvation effect (2) requires activation of one or more Crp-dependent operons under stress conditions.

Each adaptive mutation system has its own molecular mechanism: IS element movements in TIMM; Rec-, Tra- and PolII-dependent frameshifting in *lac33* reversion; and Mu A-mediated rearrangements in *araB-lacZ* fusions. What all three systems (and other examples of adaptive mutation) have in common is the activation of one or more natural genetic engineering systems capable of mediating DNA change in response to a particular kind of physiological stress. As seen in the *araB-lacZ* system, this genetic response to stress involves the control circuits that condition all bacterial responses to stress (46).

SIGNIFICANCE FOR THINKING ABOUT EVOLUTIONARY MECHANISMS

Bacteria continue to provide the most thoroughly-documented examples of evolution occuring under our eyes. They also provided the molecular evidence to confirm McClintock’s discovery of transposable elements and cellular functions capable of restructuring the genome (8, 63). These two aspects of bacterial genetics complement and illuminate each other. As we observe bacteria filling specific ecological niches, we discover

the essential roles played by intracellular DNA rearrangements involving transposable elements, cassettes and integrons, and pathogenicity islands, as well as by intercellular DNA transfer systems involving DNA uptake systems, plasmids, phages, and conjugative transposons. We have also found evidence for the assembly of major functional systems, such as catabolic pathways, adhesion systems, and protein translocation machinery by the rearrangement and joining of sequences encoding components used in a variety of contexts. From all this data, it is hard to escape the conclusion that natural genetic engineering plays a major role in what we may call bacterial “meso-evolution” (i.e. those evolutionary events that create functional complexes that confer new abilities on distinct strains within recognizable species boundaries) as distinguished from more limited “micro-evolutionary” events which alter a single protein or regulatory response (cf. 58). Given the requisite biochemical capabilities in bacterial cells (Table 2), rearrangement and diversification of basic genetic modules is a far more attractive hypothesis to explain the data than independent evolution of homologous determinants in each regulon.

Bacterial “macro-evolution” into distinct genera and species still remains largely mysterious. Genetic mapping and whole genome sequencing have revealed many differences in the genomes of bacteria and archaea. The most notable involve the organization of distinct functional determinants (clustered or dispersed) and the repetitive genome components, which differ in identity, abundance and distribution. Although we do not know the biological effects of many repetitive elements, it would be an error to assume they are non-coding. Not knowing how the sequence-specific *H. influenzae* transformation system operates, for example, we might conclude that the thousands of species identifier repeats distributed throughout the genome of this species were meaningless “junk DNA” (93, 94). Similarly, if we did not know about RecBCD-mediated recombination, we would conclude the same about the 8 bp Chi sequences distributed throughout the *E. coli* genome (which, incidentally, are not even the most frequent octamer repeats) (5). Again, it seems far more reasonable to conclude that cellular natural genetic engineering capacities

were involved in genome reorganizations and the spread of repetitive sequence elements during episodes of macroevolutionary change than that genomes diverged slowly by accumulating independent point mutations. While experimental tests of these ideas are extremely difficult because it is so hard to rule out contamination as the source of a new organism, there is an extensive older literature growing out of vaccine development on major phenotypic changes in bacterial cultures (39). It would be quite useful to revisit some of these examples of “microbic dissociation” with molecular technology.

The major mystery in evolution is the origin of novel biological adaptations, ranging from new protein functions to the formation of new structures with multiple interacting parts. Recognizing the modular, hierarchical nature of genome organization has provided a way of thinking about this problem. The reassortment of discrete genetic elements can lead to novel functions quite rapidly. The question then remains of how multiple discrete components can be integrated into workable interactive systems. A possible answer lies in two interrelated features of natural genetic engineering systems: their non-randomness and their connections to signal transduction networks. Figure 2 illustrates, for example, that many phages and pathogenicity islands insert preferentially next to tRNA and other stable RNA coding sequences. In yeast, Ty elements demonstrate a similar preference, and it is known that the Ty insertion machinery associates with RNA polymerase III transcription factors (52). The phenomenon of adaptive mutation shows that the timing and frequency of DNA changes are definitely linked to cellular physiology via signal transduction functions. Perhaps, as with Ty elements, there are also connections influencing the location of rearrangement sites. If so, then natural genetic engineering has the capacity for non-random, biologically influenced genome reorganization which could assemble novel functional systems. We should recall that cellular signal transduction networks are computational, decision-making systems (6, 7). Extending these information-processing capabilities to genome reorganization opens an entire new world to evolutionary theory.

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Table 1. Genetic "units" viewed as systems

Units view	Systems view
Protein encoded by single ORF	composite of linked domains. e.g. - α and ω domains of beta-galactosidase (LacZ) - N-terminal DNA binding and C-terminal cooperativity domains of lambda repressor - protein kinase and receptor domains of histidine kinase regulatory proteins
Gene	composite of coding sequence(s), 5' and 3' regulatory regions
Promoter (σ70)	composite of -10, -35, spacer and upstream regions with sites for positive transcription factors (e.g. Crp)
Genetic determinant of a cellular activity	combined action of coordinately regulated products of multiple genetic loci, e.g. - carbohydrate catabolism - flagellum biogenesis, motility and chemotaxis - iron uptake - pilus biogenesis - cell division

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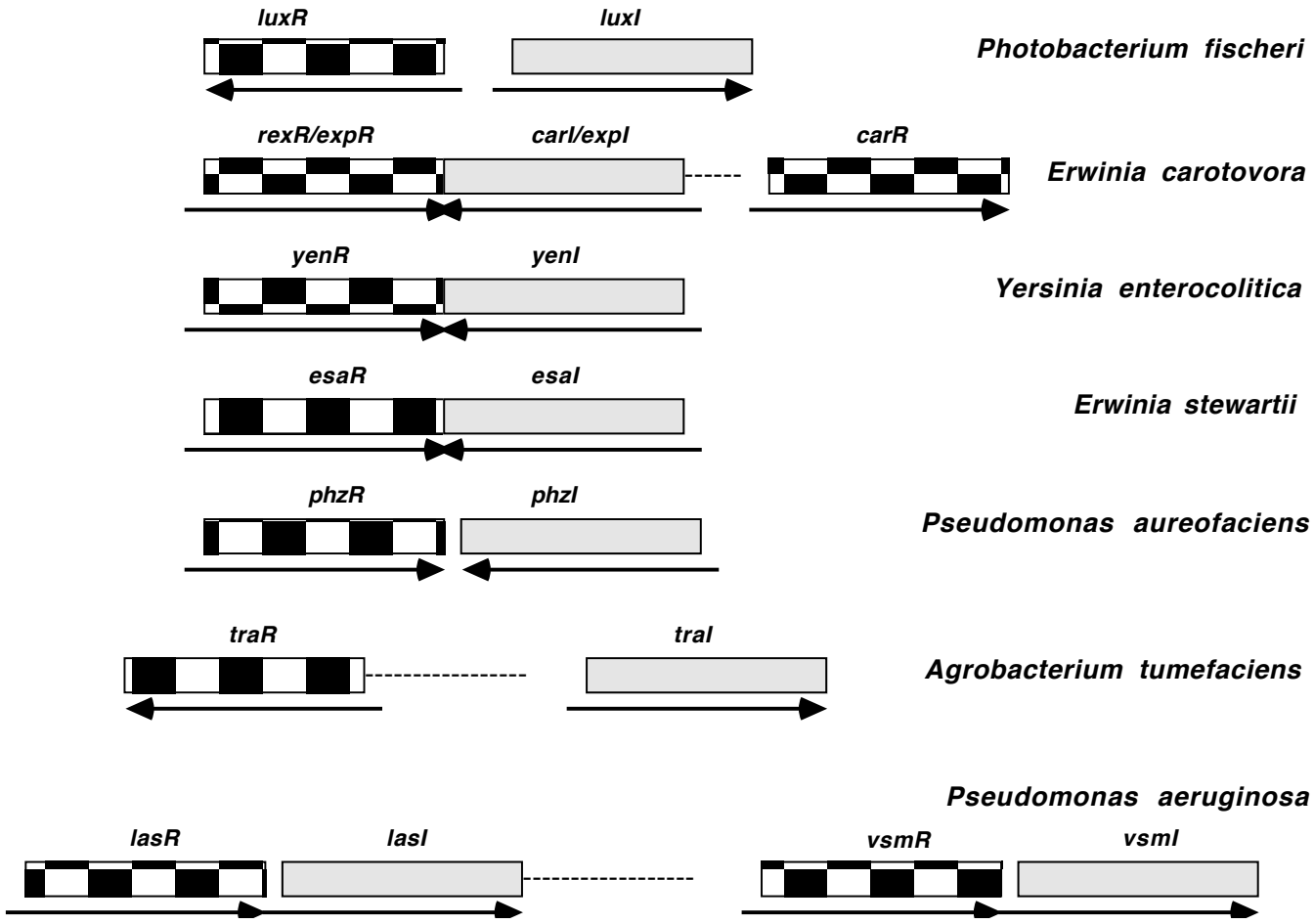

Figure 1. Arrangement of *luxR* and *luxI* homologues in different bacterial species controlling distinct functions. The arrows indicate transcripts; merged arrowheads indicate transcriptional overlaps. (Redrawn from ref. 81.)

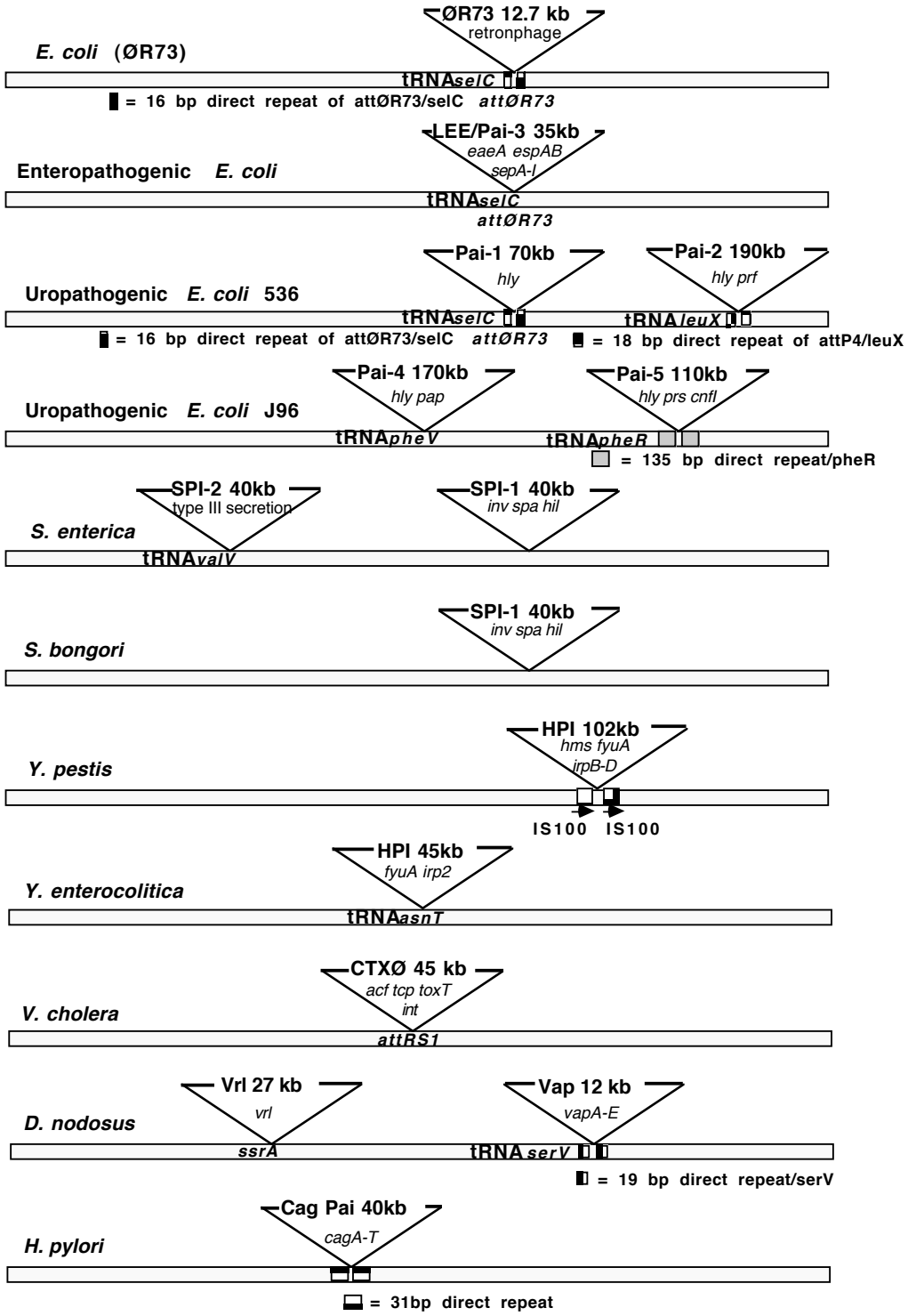

Figure 2. Pathogenicity islands (PAIs) in the chromosomes of different bacterial species. The internal lettering indicates some of the virulence functions encoded by each PAI. Based on references 34 and 38.

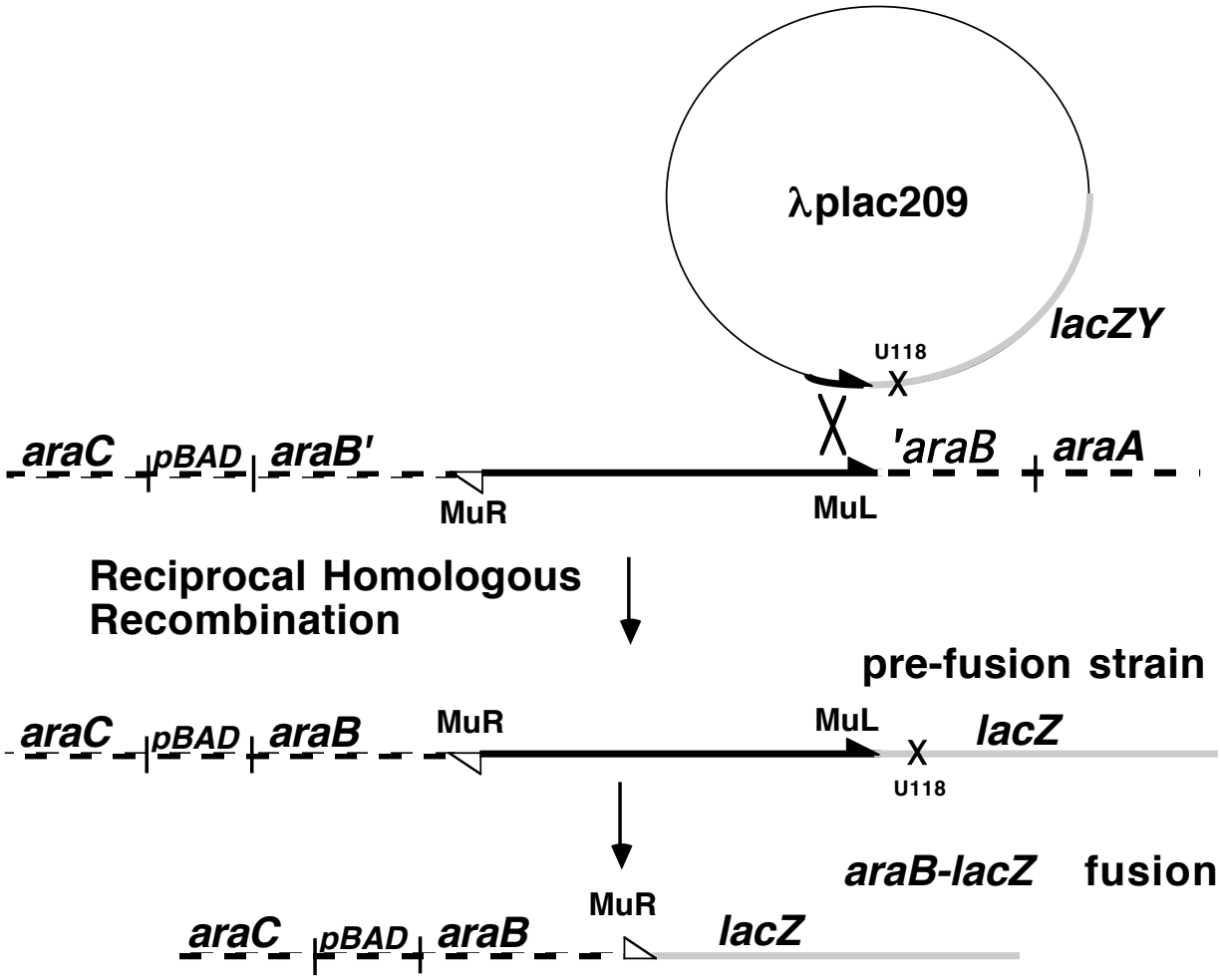

Figure 3. The Casadaban (11) system for producing *araB-lacZ* coding sequence fusions. The short MuL homology in λ*plac*209 allows the phage to integrate into an *araB*::Mu*cts62* prophage. The *lac* operon cannot be expressed because it lacks a promoter, and *lacZ* also carries the U118 *ochre* mutation at codon 17. To grow on lactose as a carbon source with arabinose as inducer on AraLac medium, an in-frame fusion must occur joining *araB* to a region of *lacZ* between codon 17 and codon 28. These fusions always carry an inverted segment of the MuR terminus (62).

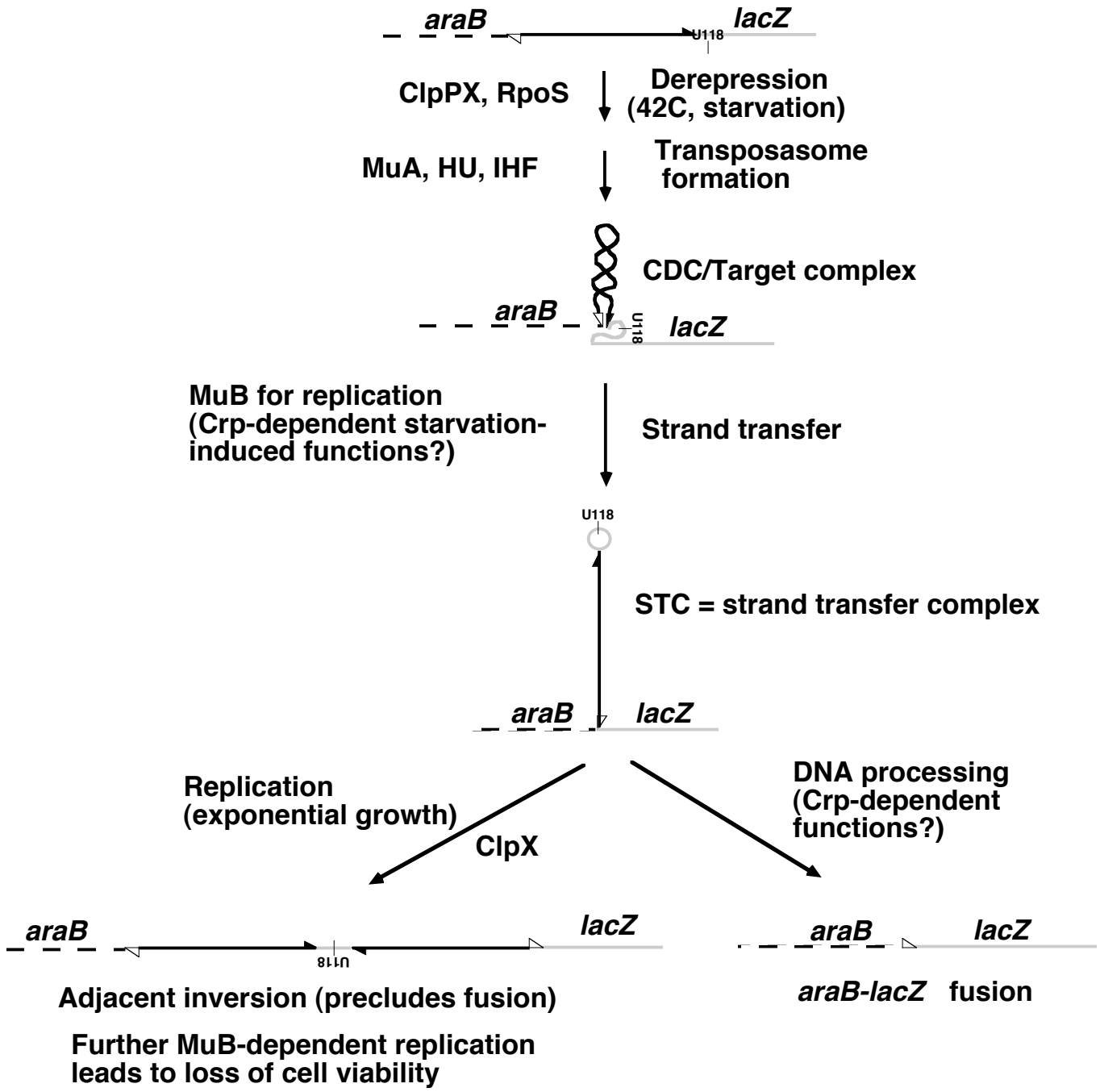

Figure 4. The different stages in *araB-lacZ* fusion formation. Inactivation of the Mu*cts62* repressor protein can occur either by incubation at high temperature or by prolonged aerobiosis at low temperature after growth has stopped. Stationary phase derepression at low temperature requires ClpPX protease and RpoS. Derepression permits Mu A expression and subsequent transposasome formation. During active growth, Mu B protein facilitates the ligation of 3' OH transposon ends in the transposasome to 5' phosphate

groups at the target site, thereby creating the strand transfer complex (STC). Mu prophage replication normally initiates after STC formation and removal of complexed Mu A protein by ClpX and other factors. Complete Mu prophage replication is incompatible with fusion formation, and further rounds of STC formation and replication are lethal to the cell. In the absence of Mu B protein, no STC appears to be formed under growth conditions after derepression, and the later steps leading to fusion formation appear to occur only under starvation conditions on the selective medium. Since the Crp protein is required for fusion formation in the presence of Mu B protein but is not essential for stationary phase derepression at low temperature, we hypothesize that it may control functions which inhibit Mu B activity and provide an alternative pathway to STC formation. Based on references 43, 62, 67, 90 and 91.