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# *Escherichia coli* and *Salmonella* 2000: the View From Here

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## INTRODUCTION

In 1995, on completion of their task, the editors of the two-volume compendium *Escherichia coli* and *Salmonella* (77) pooled their experience and wrote an epilog to that work (95). Prompted by the notable changes that have taken place in the intervening 5 years, we now attempt to update and reconsider some of what we know and don't know about these organisms. Readers should not be surprised to see that in this article we sometimes paraphrase or rely on the previous version, thus substantiating the fact that not all knowledge is new.

As before, we will refrain from making long-range predictions concerning the future of research with *E. coli* and *Salmonella enterica*. Rather, we will mention some of the immediate questions that we believe are being encountered on the way to a deeper understanding of these organisms. In so doing, we underscore why these organisms will continue to be interesting and important to study. Many of these questions will probably be answered in the near future, whereas others will prove more elusive. Surely when greater comprehension of all cells is achieved, it will be based in good part on work with *E. coli* and its cousins, since these continue to be the best known organisms on Earth. Although many other organisms have arrived at parity regarding genomic information, the game will continue to be played on this field for some time, and for good reasons.

Our choice of topics is guided by personal interests and biases. We omit or barely mention many important and exciting areas of research. Not only that, our citations are representative rather than comprehensive. We provide only a few specific citations and refer to chapters in *Escherichia coli* and *Salmonella* where older pertinent material can be found. Another disclaimer is necessary. We will not discuss *E. coli* or *S. enterica* as pathogens, as much as headlines might incite us to do. A great deal is being learned about the basic biology of these organisms from their interactions with the host. However, this is a formidable subject that merits much more attention and space than what we can devote here.

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## THE GENOME

The chromosome of the K-12 strain of *E. coli* is made up of nearly 4,400 genes. Several lines of evidence suggest that the genes required for survival under laboratory conditions (the minimal genome) are a small fraction of the total, perhaps a few hundred. Why do so many genes fall outside this category? Any extant genome represents the current version of an evolutionary tug-of-war between the selective forces of metabolic efficiency and adaptability. Efficiency impels an organism toward a small genome, whereas adaptability promotes the retention of genes required only occasionally under the diverse environmental circumstances that an organism is likely to encounter. As with all organisms, the genome of *E. coli* or *S. enterica* represents a satisfactory compromise between economy and versatility.

Seen from one vantage point, the genome of *E. coli* appears to be a model of efficiency: there are no introns and the genes are densely packed, to the extent that, by one estimate, as many as 30% of them overlap with their neighbors (12). On the other hand, why are there so many genes? The maintenance of rarely needed genes appears to come at a relatively low cost—an average gene is approximately 0.0004% of the mass of a typical *E. coli* cell. Since this figure may be insignificant, carrying a little unneeded DNA may not be a significant evolutionary handicap. So the answer may lie elsewhere. There is a great deal of evidence that the genomic composition of present-day bacteria has come about from a balance between the loss of

genes and their acquisition by horizontal transfer, both of which are potentially rapid processes (60, 72, 83). Classical mechanisms invoked for evolution in eukaryotes, point mutations and chromosomal rearrangements, act too slowly to explain what happens in bacteria. In eukaryotes, the major events in evolution have been the acquisition of microbial symbiotic genomes (retained in reduced form in various organelles).

The explosive developments in genomics have highlighted the importance of the physiological and genetic work done with our two organisms (see e.g., reference 109). Much of genomic annotation, the leap from DNA sequences to the identity of genes, has been based on information gathered over a century of work with enteric bacteria. In turn, our understanding of these organisms has advanced substantially because of facts gathered from other genomes. Central to the questions of the day is the identification of all if not most genes, both known and unknown, and their attribution to function or functions. We believe that current annotation is a complex matter, perhaps more so than meets the eye. Our presentation includes reasons for believing that the attribution of function to many of the "known" genes should be regarded as provisional for some time to come. For several websites devoted to genetic and physiological data on *E. coli* see the list at the end of this article.

At present, perhaps one-third of the genes of *E. coli* escape even tentative identification, genetically and physiologically (10, 93, 98, 111). What is hiding in the unnamed portion of the *E. coli* genome? Unadorned genetic analysis will not suffice because mutations in some of these sequences lead to no demonstrable phenotypes, at least under laboratory conditions. Many of the genes still in the annotation queue are likely to be concerned with survival in changeable natural environments, such as rivers, soil, dung heaps, foods, and healthy and diseased hosts. It will be particularly interesting to sort out which genes are common to several environmental experiences and which are specifically expressed in individual niches. In addition, how many open reading frames represent genes for functions that we have not yet surmised? Have we identified all the genes involved in higher-order cellular processes, such as cell division, nucleoid segregation, supramolecular assembly, and the orchestration of regulatory mechanisms? These are phenomena that, despite much progress, are not yet adequately understood. Some of the incognito genes are likely to be species specific, whereas others will be shared across taxonomic divides. The chromosomes of *E. coli* and *S. enterica* are quite similar in sequence and function, but about 25% of their genes are not shared, i.e., are considered to be species specific. A number of these genes have not been annotated so far, whereas others (prophages, insertion sequences, transposons, etc.) are known. With current efforts, we are optimistic that the veil over most of the unknown genes will be lifted soon.

At the time of writing, we have at hand the complete sequence of only the *E. coli* paradigm strain, K-12 (in two versions, with a third in press). Some natural isolates, including virulent strains, contain as many as $10^6$ more bases than K-12 and have diverged in other ways as well. Given the breadth of the species concept in these organisms, we expect a second explosion of genomic information from the sequencing of strains with different ecological habits. At the time of writing, the joining of contigs for about a half dozen salmonellas is close at hand.

We can expect to enhance our ability to deduce the function of gene products from sequence analysis, including the recognition of motifs and domains. However, we already know that this is a subtle and sometimes deceptive business. Small variations in sequence may have a drastic effect on the substrate specificity of an enzyme. Conversely, having the same function may not be due to kinship in sequence, as illustrated, for example, by phosphoryl transfer enzymes, such as RuvC, RNase H, and the transposases. All these enzymes have the same catalytic core architecture even though their primary sequences are quite different (26). A great deal of sophisticated computational work lies ahead. However, we assert again that this is likely to prove insufficient in the absence of detailed physiological information regarding the diverse environments and stresses that a particular bacterium encounters. This point has been discussed in the context of the redundancies in the thioredoxin superfamily (5).

Predictably, there will be continued interest in special aspects of the organization of the genome such as genomic rearrangements (92) and special elements such as repeated sequences (6), cryptic prophages (17), and insertion elements (28).

Does some DNA play an architectural, noncoding role? Answers should be forthcoming as microbiologists and computational scientists come together to develop the methods to perform and analyze whole-cell gene expression profiles by using DNA arrays (see, for example, reference 3).

## EVOLUTION

We can expect that the analysis of genomic sequences will continue to have a weighty effect on our understanding of microbial evolution. The study of evolution has been late in coming to microbiology, surely not just for lack of an extensive fossil record, but is now enjoying a hearty awakening. With this way of looking at bacteria becoming so ingrained, it is difficult to guess what will be singled out as evolutionary research in the future. We can point to a few topics that are likely to have a particular influence in this field. The increased interest in gene transfer between species opens the door for new areas of inquiry. This perspective colors the basic thinking on evolution in the microbial world and elsewhere. It emphasizes that evolution is work in progress, and some of what we observe now may well represent decaying genes or other elements whose presence does not necessarily reflect the most adroit configuration imaginable even for today.

Over half of *E. coli* genes and proteins fall into sequence-related families. The most frequent form of kinship among these proteins is pairwise, but the landscape is dominated by a few very large groups of relatives, mostly transporter and regulatory proteins (58). One explanation for the existence of sequence-related families is that ancestral genes duplicated and then diverged in function but remained close enough in sequence to permit similarities to be detected. Some duplications may have occurred at early times in evolution, as suggested by the presence of proteins of likely common ancestry in many species. Other duplications may have occurred over time, even up to the present, thus generating families of genes and

proteins that are unique to certain related species. Some of these phenomena are accessible to experimental studies. Rapid sequencing techniques will allow one to measure the divergence of duplications in populations maintained for thousands of generations under defined conditions and to correlate it with fitness (see, for example, reference 90).

The role of horizontal transfer of genes between species has been emphasized in recent years. One analysis suggests that about 17% of the *E. coli* K-12 chromosome is derived from unrelated outside sources (60). Over half of the identified genes belong to transposons, temperate phages, and the like and are thus out of evolutionary step with the rest of the chromosome.

Comparative studies of enzymes of pathways (82) and the DNA sequences of homologous portions of the chromosome (71) have shed light on what kinds of molecular changes constitute evolutionary movement and how recombination and mutation affect population dynamics. There is a surprising conservation of certain patterns across genera (see, e.g., reference 120). Still, the functions of rearranged genes in one bacterial lineage may not be the same as in another. The relationship between these evolutionary clues and cell physiology has yet to be fully explored and remains yet another exciting frontier.

Bacterial evolution is vitally intertwined with the enchanting world of plasmids and phages. Of the many examples available that illustrate the might of plasmids, we single out two, a novel mechanism whereby plasmid-located genes silence some of their own genes (113) and the ways that certain wily plasmids have developed to ensure that their host cells carry them along (33, 39). The phenomenon that motivates bacteria to hold on to these plasmids lest they perish has been fittingly called addiction. There are several themes here, since individual plasmids cause addiction via a variety of different mechanisms (112). Likewise, temperate phages clearly play a central role in bacterial evolution (17). We can expect that the plasticity of the bacterial genome, in its various guises, will be an important component of evolutionary studies. The richness of this subject will continue to captivate its students.

## MOLECULAR MULTIFUNCTIONALITY

Some of us are old enough to remember the lemma "one gene, one enzyme," which influenced much of early molecular biology, but it has been long time since we have heard it used. There were many reasons for relinquishing it, including the discovery of partnerships such as "one gene, four enzymes," "two genes, three enzymatic reactions," and other unorthodox-sounding combinations (91). Much of this complexity arises from the fact that many bacterial proteins are multifunctional, i.e., can be involved in more than one physiological activity. We use the term "multifunctional" in the broadest sense, not the one biochemically restricted to proteins with more than one catalytic domain.

As recently emphasized by Hartwell et al., (38) and Alberts (2), complex cellular functions are carried out by modules made up of interacting macromolecules. A given protein may contain multiple modules and thus be involved in several functions. Alas, such intricate reality does not make it any easier to accomplish genomic annotation. Evidence for widespread multifunctionality not only derives from biochemical studies but also is suggested by computational analyses indicating that each protein of *E. coli* is likely to interact with 2 to 10 other proteins (31, 68). An experimental approach to this question on a large scale is forthcoming owing to the recent introduction of protein microarrays (64).

Proteins may do more than one enzymatic job, as with the classic examples of penicillin binding protein 1B (MrcB), which has both transglycosylation and transpeptidation activities, and RecA, which can function either as a recombinase or as a coprotease. Because the domains of some of such proteins are found as solo entities in other species, the multifunctional versions probably arose by gene fusion. Specific domains may also be shared by different proteins to create distinct functions (this is known as domain shuffling). An example is the LysM domain, which in *E. coli* is part of the otherwise unrelated proteins murein transglycosylase D and the host cell-adhesive protein intimin (8).

Some multiple functions cut across physiological classes, as was pointed out some time ago (51). A classic example is phage Qβ replicase, which consist of four subunits, only one of which is phage encoded. The other three recruited subunits are host proteins involved in protein synthesis, namely, ribosomal protein S1 and elongation factors EF-Tu and EF-Ts (11). Certain proteins that have a catalytic activity also act as a regulatory device, as seen, for example, in the enzymes that control their own synthesis. Some proteins, e.g., some ribosomal proteins, act as both structural components and regulators (52). One protein, PutA, is known to have an enzymatic function when incorporated into the membrane and a regulatory role in its soluble form (73). We envisage that in the future, the table of the proteins of *E. coli* and *S. enterica* will include a list of the manifold functions known for a large proportion of all genes and proteins. This is likely to come from the congruence of genomics and proteomics and the increasingly sophisticated use of physiological and genetic techniques (114). The finding of enolase in the degradasome complex involved in mRNA degradation (85) prepares us for future surprises and puzzles in such a table.

Proteins can be expected to vary in the number of distinct physiological functions that each possesses, with some proteins being quite multipurpose and others being single-minded. There is a need here to sharpen definitions. A pragmatic distinction between "activity" (substrate binding, catalysis, etc.) and "physiological function" is useful. For example, DnaA protein has a number of biochemical activities (it is a sequence-specific DNA binding protein and also binds nucleotides, phospholipids, and DnaB protein), but it also participates in a number of different physiological functions (it both organizes and regulates initiation of DNA replication, as well as acting as a repressor, terminator, and enhancer of transcription of a number of genes [70]). These two levels not only are causally related but also, in many cases, cannot be readily demarcated.

We must respect the reality that a genetic approach alone may lead to equivocal conclusions. Multifunctionality in proteins means that mutations in their genes are pleiotropic, which makes a simple interpretation of their phenotypes difficult if not misleading. As more attention is paid to the study of phenomena of greater complexity, other limitations to a purely

genetic approach are encountered. One obvious reason is that complex functions are likely to depend both on specific "dedicated" systems and on nonspecific factors that respond in a general way to environmental changes. Mutations in either class may lead to defective phenotypes. Additionally, the response to virtually any stress requires cooperative effects of specific and general homeostatic processes.

## BACKUPS

The other side of the coin to protein multifunctionality is the existence of backup systems for individual functions (examples for nitrogen metabolism are found in reference 66, and examples for rRNA are found in reference 52). Genetic redundancy results in the noticeable number of mutations in apparently important genes that do not lead to cell death. When it becomes known that this is due to the presence several copies of related genes, strains with multiple mutations must be painstakingly constructed. This approach has proven useful in the study of penicillin binding proteins (27) and rRNA genes (4). For backups involving unrelated genes, a useful technique may be the construction of synthetic lethal mutants of a kind (whereby the gene in question can be conditionally turned on or off, thus allowing the search for mutations in backup genes). However, such approaches must be used with care and should be used only to suggest further experiments rather than to draw strong conclusions about backup functions. Greater attention will have to be paid to the interpretation of mutant phenotypes and to the search for alternate defects than the one originally used for selection or screening.

What do functional redundancies tell us about bacterial evolution? Obviously, if two proteins carry out the same function, one must be the older one, but does this mean that a newer, better-evolved one has superseded it? If so, why does the organism carry along the vestigial system whose activity is rarely called upon? At first glance, such systems may appear to be economically demanding, but it is likely that backups usually recruit proteins that are probably serving other functions for the cell under other conditions. Redundancy may not be the right word, in the sense that each variant probably plays different physiological roles. Hence, most backups have probably not evolved de novo as dedicated systems but are already present in the cell for other reasons. Examples are the use of the recombination system RecA, RecB, RecC, and RecD, which effects initiation of chromosome replication when the normal machinery is inert (70), and the numerous sets of seemingly redundant functions in the various pathways of homologous recombination and repair (62). We know that when there are several enzymes for the same function, their genes are often differentially expressed according to environmental circumstances. Enzyme activity, substrate specificity, and response to inhibitors or activators may vary depending on the conditions (87). This suggests that seemingly redundant proteins may be valuable in expanding the ability of the cell to respond to a wide range of environmental circumstances (5).

## REGULATION OF GENE EXPRESSION

Studies on gene regulation have dominated basic enterobacterial biology for nearly five decades. Is the end in sight? Not really. . . and there are significant indications that some old quests may continue even as long and new ones begin. The repertoire for regulating gene expression in *E. coli*, already impressive, is likely to grow as more and more of the newly discovered genes are analyzed. Along with the complete sequence of the *E. coli* genome, there are the new tools that permit monitoring of both the transcription and translation of each individual gene.

Our current understanding of gene regulation in *E. coli* suggests three hierarchical levels of control (in increasing order of comprehension): (i) global control by chromosome structure, (ii) global control of stimulons and regulons, and (iii) operon-specific controls.

The first level of control is exemplified by DNA-supercoiling-dependent mechanisms that coordinate the basal level expression (independent of operon-specific controls). Operating here are the local superhelical density and the topological structure of the whole chromosome. An example is the role of supercoiling in the expression of the operons of the *ilv* regulon (100). DNA architectural proteins, such as integration host factor, modulate the formation of these structures. The second level of control is mediated by site-specific proteins, which, in cooperation with operon-specific controls, regulate often overlapping groups of metabolically related operons in response to environmental or metabolic signals. The third level of control is mediated by less abundant regulatory proteins that respond to operon-specific signals and bind in a site-specific manner to one or a few sites to regulate single operons.

Regulation at the level of enzyme activity has also received its fair share of attention, permitting satisfactory connections between older and more recent findings. At this level, the elegant studies from Atkinson's laboratory taught us long ago that the energy charge of the adenylate pool is an important global parameter of metabolic regulation (19) (the energy charge is [ATP] + [ADP]/[ATP] + [ADP] + [AMP] rather than the ATP concentration alone). When cells go from aerobic to anaerobic growth, the energy charge transiently decreases, enzymes involved in ATP-regenerating reactions are activated, and enzymes involved in ATP-utilizing reactions are inhibited. Thus, increasing the activity of enzymes that produce ATP and decreasing that of enzymes that consume it counteracts any tendency for the energy charge to fall (20). These findings explain how the cell coordinates energy metabolism with the anabolic and catabolic reactions of the cell.

The link between energy charge and global gene regulation has been clarified by finding that the global level of negative DNA supercoiling levels is maintained by DNA gyrase (118). And, of course, DNA gyrase activity is dependent on the energy charge of the adenylate pool. Thus, changes in the cellular [ATP]/[ADP] ratios correlate with changes in DNA supercoiling (29, 45, 49, 118). DNA supercoiling plays a functional role in cellular adaptation and survival under various suboptimal growth conditions. Well-characterized examples include gene expression changes that occur during stationary-phase growth (57), osmotic stress (42), and aerobic to anaerobic growth transitions. On a broader scale, topoisomerase mutations that cause in vivo changes in DNA supercoiling affect the levels of expression of a large number of proteins (105). Of the 88 proteins that were quantitated on two-dimensional gels, 39%

showed changes of abundance (and, inferentially, of expression level) during steady-state growth in *topA* or *gyrB* strains.

Regulation of gene expression not only is a protein affair but also involves nucleic acids in unexpected ways. Increasingly, RNA molecules have been found to participate in regulation. It is particularly interesting that distinct phenomena are involved, such as the processing of RNA by RNase $P_1$ (13); the negative and positive actions of antisense RNA (116); the targeting of proteins for degradation by SsrA, the product of tmRNA (10Sa RNA [74]); and the regulation of protein activity by 6S RNA (117). These are only a few examples in a rapidly expanding field.

Unanticipated aspects of bacterial regulation do not stop there. Some bacteria, notably *Salmonella* and *Yersinia*, regulate gene expression by ridding themselves of regulatory proteins—they secrete them from the cells. In *E. coli*, FlgM, a protein that inhibits a sigma factor specifically involved in flagellum synthesis, is thought to be exported through the hollow hook-basal body on assembly of this structure (14). Are such regulatory purges restricted to the use of flagella and type III secretion structures, or are other examples waiting in the wings? More generally, do cells make much use of "allolocation" (John Roth's apt term to indicate that the activity of a regulator depends on its location)? We have only a few examples so far, the ones mentioned here and that of the PutA protein (73).

Such accumulation of information will not merely lead to a catalog of mechanisms but, rather, will invite examination of how such devices are harmonized during cell growth. Thus, we expect that increased attention will be given to higher level integration of control mechanisms, and terms such as "network" and "cross talk" will come into increased use when considering the regulation of the living cell. A pioneering example is the analysis of the lytic-lysogenic choice in phage lambda by McAdams and Shapiro (69). Although regulation of gene networks is a topic still in search of new paradigms, this is not to say that our current knowledge lacks sophistication. There are many cases in which significant understanding of the complexity of a regulatory system has been achieved. For example, the individual operons of the Crp regulon differ quantitatively in their response to their corresponding regulators (21, 96). The Lrp regulon is highly modulated by the recruitment of accessory regulatory proteins and the differences in sensitivity of individual operons (78).

Studies of bacterial responses to stress have become a major theme in the traditional realm of bacterial physiology. How the environment is sensed, what signals are generated, how these are transduced, how the ultimate cellular response is effected, and how the response is controlled are being studied with verve and effectiveness for dozens of environmental stresses (106). Elucidating the communication between environment and periplasm and between periplasm and cytosol is well under way (86).

The subject of stress has recently taken a fascinating turn. For almost two decades, the *E. coli* responses to temperature and pH changes, oxidative threat, heavy metals, osmotic stress, and radiation-damaged DNA have been studied as separate and distinct physiological processes. In contrast, stress responses have now become intertwined not only with each other but also with growth physiology and differentiation in general. We now know that no stress response is an island. As dramatically revealed by global monitoring of the transcriptome and the proteome, the response to any significant stress involves adjustments in the rates of synthesis of hundreds of different proteins beside the specific responders. The ancillary responders include two large and important sets of proteins. One set consists of proteins whose level is adjusted to match the growth potential of the given environmental circumstance (e.g., the nearly 100 proteins of the translation and transcription machinery of the cell). The other set consists of the vast host of proteins involved in transforming the structure and metabolism of the cell from a growth mode to a survival mode. Induction of the latter set is facilitated by the alternate sigma factor, $\sigma^S$, the product of the *rpoS* gene. Our understanding of the physiology of $\sigma^S$ now goes far beyond the notion that it is a key regulator in the stationary phase (which it is) to include the realization that it is a central player in what is now termed the general stress response of *E. coli* (41). Studying how this sigma factor functions in concert with other global regulators and how it itself is controlled will lead us deep into the complex circuitry of the entire gene regulation network. Beside the proteins related to growth physiology and to the general stress response, still other stress responders are involved in repair of damaged cell structures, depending on the severity and duration of the stress.

Finally, the interconnectivity of the stress response systems has a further significance. One can imagine that under many circumstances the *E. coli* cell is struck not by one but by two, three, or many more simultaneous stresses. Certainly this occurs during a most normal (but potentially calamitous) event in the natural history of *E. coli*, exiting the host. How the cell handles the simultaneous challenges of cold shock, starvation, and changes in redox potential, osmolarity, and dilution will undoubtedly be an experimental subject in the near future.

With so many ways of regulating the synthesis and activity of proteins, how does the cell decide which is the most appropriate to the task? Is each uniquely suited to its role? The groundbreaking studies on the differential operation of end product inhibition, repression, and attenuation in the regulation of tryptophan biosynthesis (59) point the way. So do the studies of Savageau (76), which employ a technique called power-law formalism to represent mathematically the dynamic behavior of intact biochemical systems. This approach has already uncovered several rules that successfully predict the mode of regulation selected in nature for gene regulation in different circumstances. For example, one simple rule is that regulation occurs by a positive mode when there is a demand for high levels of expression of the regulated gene in the natural environment of the organism and by a negative mode when there is a demand for only low levels. This rule has been confirmed in more than 60 tests involving a dozen different types of physiological function (76). Progress in this area is doubly important (or important raised to some power!) because every time the selection of a particular molecular mechanism is shown to have a rational basis, the attention that will be paid to studying system behavior increases.

Evolutionary considerations may well be another source of useful hints about regulatory matters in *E. coli*. The extensive studies on the biosynthesis of aromatic amino acids (50, 79) have already disclosed the varieties of pathways that exist among different bacteria. Also illuminating are studies on *lac*

and *gal* repressors. In *E. coli* these are very similar, and thus have probably evolved from a common ancestor, whereas the enzymes encoded by these operons are quite different (21, 88). This suggests that regulation has evolved after the biochemical pathways, a situation consistent with the view that the mode of regulation is not haphazard but has evolved under the particular selective pressures of the environmental niche of an organism.

The increased availability of information on the patterns of gene expression under various conditions will guide both hypothesis and experiment. Our understanding of cellular regulation will be greatly influenced by past and present physiological studies coupled to genomic, microarray, and computational technologies. The limiting element will most certainly be our ability to analyze and express such data, and it will require the concerted efforts of computational scientists working with physiologists and geneticists. A pessimistic view (to which we do not subscribe at present) is that the integrated network represented by a growing cell is so complex that it has some properties of chaos systems and therefore its analysis will be forbidding. We think it more likely that this will turn out to be far from a hopeless task. We are awed by the ability to study the changes in behavior of all genes of an organism. However, to be ultimately useful for the understanding of the "real" organism, as opposed to the domesticated laboratory version, the conditions chosen for study will have to mirror the natural environments as closely as possible. Within the limits of our comprehension, we may be in sight of the holy grail of molecular biology, i.e., to understand the integrated genetic and metabolic patterns of an organism. That organism will probably be *E. coli*.

## HETEROGENITY IN POPULATIONS

Are populations of *E. coli* or *Salmonella* homogeneous, even in the laboratory? One of the reasons why it is so satisfactory to work with bacteria is the assumption that we are studying large populations of identical cells. For most purposes this is true but, on close examination it turns out that individual cells may differ measurably in important physiological properties. A number of examples are presented in a review by Koch (55). In most cases the variability is dampened out as growth proceeds, but in others it can lead to significant epigenetic changes and even to clonal heterogeneity. A heterogeneous population may result from frequent mutations, such as those derived from genetic inversions or insertions. Mutations of this sort occur in both directions as often as once per $10^3$ cell divisions. Examples are phase variations in flagella, with important consequences for pathogenesis, and expression of cryptic operons such as *bgl* (89).

Heterogeneity in a population may also originate from epigenetic changes (i.e., those not involving genomic coding alterations). The best-known examples involve DNA methylation, which influences gene expression in bacteria and transposons and can have profound phenotypic effects. In phages, the state of DNA methylation may alter the host range ("host modifications"). In bacteria, methylation may determine phase variation in pili and other surface components, which, in turn, may affect virulence or biofilm formation (25). The molecular basis for switches in pilus biogenesis is known and involves, among other things, the binding of Lrp protein to the methylation sites on promoters and the temperature-dependent activity of H-NS protein (80). There are indications that such phenomena occur with greater frequency than is sometimes recognized. It should be kept in mind that heterogeneity in cell populations might represent a significant source of experimental variance in the results obtained, for example, from gene-profiling experiments.

## CELL BIOLOGY

The interior of *E. coli* is not a bag of enzymes, ribosomes, and DNA, but until very recently, this was just a truism. From biochemistry alone we learn that enzymes in a pathway are likely to be found in complexes, like beads on a string, thus facilitating the transfer of reactants. We are now entering a new phase, where we increasingly appreciate how the whole bacterial cell consists of specialized and interacting compartments. A series of findings, some totally unexpected, have brought us much further along in understanding the spatial relationships of cellular components. These developments are based on refinements in fluorescence microscope technology that allow us to determine the intracellular location of specific proteins or DNA sequences. Of particular importance is the ability to observe the dynamic and spatial behavior of molecules in living cells. Indeed, in this arena a picture (or video clip) is worth a thousand words. The results have been stunning: a whole set of macromolecules occupy special sites in bacteria and exhibit dynamic behavior. These include proteins involved in cell division, chromosome segregation, differentiation, motility, and chemotaxis (99). In this field, *E. coli* faces severe competition from *Caulobacter crescentus* and *Bacillus subtilis*.

One of the main areas of focus has been on molecules involved in cell division and chromosome partitioning. The exciting finding of the equatorial localization of a protein required for cell division, FtsZ, provided a molecular mechanism for septum formation and suggests the existence of a bacterial cytoskeleton (63, 67, 75). The central question remains: how does the cell know where its middle is? We have only hints about the way FtsZ and other proteins find the center of the cell. A model suggesting that the site of septation is limited to the non-nucleoid-containing areas has been proposed (Woldringh's nucleoid exclusion model [63]). Proteins of the Min system appear to be involved in such site recognition, although little is known about how they carry this out. On the other hand, direct observations of MinC and MinD proteins in the living cell have disclosed a startling behavior. These two proteins together alternate their location between the two poles of the cell at a surprising speed of less than 1 min (87). The reasons for this puzzling molecular ping-pong are not yet known.

The term "bacterial mitosis" is gaining in popularity, implying faith that we are beginning to understand how it works. Current knowledge suggests a mechanism quite different from that proposed originally for the replicon model. For readers who are new to this field, the replicon model proposed that the replicative origins are attached to the cell envelope and that progeny chromosomes separate by the zonal growth of the envelopes. Early on, it turned out that the envelopes do not

grow in this fashion but, rather, grow by multiple intercalation (for evidence on the cell wall, see reference 15). A more recent problem with the model is the finding that the origins of replication move apart during replication to occupy sites near the cell poles (35, 99) and remain there for most of the cell cycle. The replication machinery, the replisome, appears to be located at the cell center, presumably attached to the cell membrane (63). Thus, to carry out a new initiation, the origins must move back to the cell center. How do they do it?

As unexpected as the behavior of the origin is the finding that the nucleoid is not a disorganized ball of twine floating in the cytoplasm. Rather, it appears that the position of genes along the chromosome is reflected in their cellular location. For most of the cell cycle, genes nearer the origin on the genetic map tend to be located near the poles and those farthest away tend to be located toward the cell center (81, 110) Because bacterial DNA is over 1,000 times longer than the cell, this finding suggest that the chromosome consist of loops that are positioned in an orderly fashion with respect to the topology of the cell. We do not know whether these findings relate to earlier ones on chromosome structure. The chromosome appears to be divided into 50 to 100 individual supercoiled domains, so that relaxation of one does not lead to the relaxation of the whole molecule (84). Similar values have been calculated for the number of attachment sites of the chromosome to the cell membrane. Other studies have also pointed to the existence of barriers to supercoil diffusion (104) and, over a longer range, to recombination (T. Harmon and A. Segall, personal communication). What holds domains intact is not known, although various possibilities have been invoked, such as the location of gyrase molecules or the attachment to the cell membrane either directly or indirectly by the insertion of nascent membrane proteins. Other suggestive local phenomena are also known, such as the distance effect of Tn*7* insertions, known as local immunity, which limits the insertion of other Tn*7* molecules over a distance of at least 190 kb (23). Likewise, recombinational events involved in the resolution of chromosomal dimers take place only when the loci involved are near the replicative terminus (16).

How, then, are progeny nucleoids separated into the progeny cells? It has been proposed that the act of partition is associated with DNA replication. As the DNA passes through the replisome, it loses supercoiling and becomes decondensed. Once replicated, the chromosomes are separately recondensed and move towards the 1/4 and 3/4 positions of the cell (see reference 44 and references therein). Involved in the condensation is a bifunctional protein, MukB, which is a member of the SMC family (for "structural maintenance of chromosomes"). These proteins are involved in DNA condensation and other aspects of chromosome structure. Each of these two-headed protein molecules contracts DNA by binding to two distant sites and pulling them together by a hinge-like mechanism. ATP is used in the process.

Compartmentalization within a bacterial cell is also suggested when considering the regulation of gene expression. In many cases, the number of molecules involved is of the order of a few dozen per cell (e.g., the *lac* repressor). In this regard, old questions are still with us. How do a few regulator molecules regulate? (Analogously, how do protein molecules that complement others find their partners with such great speed?) Are extra regulatory molecules titrated away by being sequestered at multiple target sites? A surprising number of proteins appear to be "bunched" in foci within the cell rather than being broadly distributed (43, 99). The nascent field of biochemistry at the single-molecule level may be rewarding here (115). There are also exciting prospects for studying kinetics at the single-cell level, which allows the precise calculations of protein concentration in individual cells in order to correlate it with their behavior (22).

The frequency with which a single molecule present in small numbers collides with its target may not be dictated by the law of mass action alone. This point was made explicitly in 1994 by John Maddox in a discerning article (65). It is appealing to think that proteins that bind to DNA do not encounter their binding sites just by freely diffusing through the cell. Rather, the idea has been put forth that proteins find their particular targets on the DNA by tracking or sliding, binding initially at one entry site and moving along the DNA until they encounter their target (88, 101). Is spatial proximity of nascent proteins the answer in some cases? Regulator molecules do not necessarily appear to be made in *cis*, "close" to their targets, as is the case with certain transposases. Additionally, DNA and probably other macromolecules are influenced by the nature of the solvent, which compacts them by providing aqueous phase partition. This phenomenon, called molecular crowding, diminishes the effective space over which molecular interactions take place (84). Such considerations add credence to the notion that the interior of *E. coli* is something utterly different from a random gemisch of macromolecules.

## SENSING OF THE ENVIRONMENT

How are physical signals translated into chemical activity? How do proteins act as thermometers, pH meters, osmometers, "piezochemical" molecules, etc. (24, 48, 102)? In some cases, considerable understanding has been achieved already. An example is the protein FNR, which is endowed with a [4Fe-4S] cluster that acts as a sensor of the oxygen concentration (53). Will some of the signal transduction systems find widespread commercial applications as ultramicrotechnological sensors?

We know that environmental signals alter such fundamental cell properties as the ADP/ATP ratio and the superhelical density of the chromosome. As we have mentioned above, the expression of large numbers of genes is affected by such changes.

Bacteria communicate with one another through low-molecular-weight compounds, a phenomenon called quorum sensing. In many species, homoserine lactone derivatives are the signaling device used when cultures enter the stationary phase, whereas in other species peptides are used for this purpose (107). In the case of *E. coli*, the signaling compounds have not yet been identified, but, interestingly, at least one of them appears to down-regulate genes involved in cell division (34). In *Salmonella*, a homologue of the gene involved in *E. coli, SdiA*, regulates genes on a virulence plasmid, suggesting that quorum sensing may be implicated in pathogenesis (1). We anticipate that the study of such metabolic signals will help elucidate how bacteria cope with environmental changes by using intercellular interactions. There is already evidence that

quorum sensing modulates cell-cell communication not only within the members of a species but also between species. In ways analogous to social insects, bacteria use chemical communication and sensing to establish complex communities such as biofilms (7).

In the field of sensory transduction, an open question relates to the frequent use of protein phosphorylation as a regulatory device. Little is known about how kinases that are relatively nonspecific manage to phosphorylate certain proteins and not others (36). In some two-component systems, the kinase can be dispensed with and its activity can be substituted by the phosphate donor ability of a common metabolite, acetyl phosphate. The reason acetyl phosphate does not usually perform this role is that certain kinases act as phosphatases, destroying this biochemical impostor. This is an unanticipated example not only of functional redundancy but also of the hegemony of one pathway over another.

An intriguing form of intercellular communication appears to lead to programmed cell death when growth is inhibited. An addiction module of the sort found in plasmids has been found in *E. coli* (30) and consists of a stable protein "toxin" and an unstable "antitoxin." When transcription or translation is halted, the toxin continues to be active but the antitoxin decays, resulting in cell death. The mediators of the stringent response, polyphosphorylated guanosines, are involved, apparently by downregulating a promoter for the antitoxin gene. An extracellular factor appears to be involved in the process of programmed cell death.

It is with regard to sensing the environment that studies with pathogenic strains are coming to the fore (32). It is becoming increasingly clear that signal transduction mechanisms allow bacteria to "know where they are" and to express different sets of genes within and outside the host. Techniques that allow one to identify genes that are used for survival in the host may well sharpen and broaden our definition of virulence factors (18). Sensing the host environments has led to the discovery of remarkable mechanisms for the establishment of infection. Prominent among them is type III secretion, a highly regulated process that introduces bacterial proteins into host cells subsequent to direct cell-cell contact (37). This process is doubly regulated. As deduced from diverse organisms, the genes of such regulons are first turned on by relatively simple environmental signals, such as vertebrate body temperature. This then leads to the assembly of a structurally complex secretory machine related to basal components of flagella. Second, contact with the host cell induces the secretion into the host cell of effector proteins that foster stronger adhesion of the organisms or their phagocytosis. This is a superb example of nanotechnology in the microbial world, one that may well be exploited by humans for the delivery of drugs into specific host cells.

## TECHNIQUES FOR STUDYING THE LIVING CELL

We expect to see the increased development and use of existing methods that allow us to come closer to studying the living cell. Some of these techniques already allow observations in growing cells, whereas others that operate at the whole-cell level still require manipulations that kill or disable the cells. The extent to which these interventions modify the cell contents is generally not known, but it is expected that the use of several techniques in combination with each other and linked to genetic and biochemical tools will help elucidate important cellular processes.

Overarching the technology panorama is, of course, the use of DNA and protein microarrays and similar technologies, coupled by novel uses of analysis such as mass spectroscopy. The three-dimensional structure of most *E. coli* proteins may well become known before long. Although these techniques do not probe living cells directly, they point out priorities for in vivo studies.

In vivo techniques and manipulations, in different stages of development, include (i) Further applications of fluorescence and other forms of microscopy in conjunction with the development of specific tagged probes, (ii) methods to make cells permeable to externally added proteins, (iii) in vivo DNA footprinting using synchronized and nonsynchronized cells, (iv) in vivo cross-linking of various classes of molecules, (v) in vivo nuclear magnetic resonance spectroscopy to measure metabolites, and (vi) Raman and other forms of spectroscopy to detect specific molecules.

In addition, flow cytometry allows one to measure not only cell volume distributions but also the distribution of macromolecules in populations. This technique will benefit from the use of novel probes for labeling individual proteins and other molecules or, particularly exciting, to detect special configurations of nucleic acids and proteins.

Although methodological innovations frequently result in scientific breakthroughs, this is not invariably the case. Being mindful that new techniques can be particularly seductive, we remember the admonition of Salvador Luria, who said, "If something is not worth doing, it's not worth doing well."

## THE REAL WORLD

It is becoming increasingly popular to reflect on physiological experimentation from the vantage points of evolution, population genetics, and ecology. We believe that this outlook will continue to occupy center stage. However, bacterial physiologists, ecologists, and population geneticists do not always speak the same language. To the physiologist, *E. coli* is a master of efficiency, carrying out its physiological business, i.e., growth, with dazzling competence. An *E. coli* cell growing in artificial media directs its biosynthetic activities to the making of the metabolites or macromolecules it can use at the moment. When the in vitro environment changes, *E. coli* makes swift and efficient adjustments that favor its survival. Bacteria such as this one possess an impressive array of sensing devices that tell them when they are starving; when the temperature, pH, or salinity changes; when inhibitory substances are present; and when their DNA has been damaged and needs repair.

To the ecologist, *E. coli* is a minority component of the normal biota of the large intestine of vertebrates, occasionally dwelling in soils or bodies of water. Only during disease and only with certain strains does *E. coli* exceed 1% of the total number of bacteria in feces. Strict anaerobes dominate in the lower gastrointestinal tract, and our hero is relegated to a much lesser role than its laboratory prominence might imply. Our knowledge of the enormously complex environment of the vertebrate gut is still rudimentary. Microbial ecology varies

along the length of the gut, with different organisms growing freely in the lumen and others living in the mucus layer or attached to the mucosal surface. The microbial ability to adhere to the intestinal wall is a key determinant of health and disease (47).

Many "real-world" questions arise. Why does each one of us carry his or her own repertoire of strains? Why is the population of *E. coli* renewable? Is this balanced situation achieved in response to nutrient limitation, is it due to the effects of inhibitory substances, or both? It is not widely appreciated that *E. coli* is a dominant organism in the relatively sparse biota of the lower small intestine, where it can possibly grow unhampered under aerobic conditions and with a rich supply of nutrients (54, 94). From the *E. coli* point of view, does this part of the intestine resemble well-aerated Luria-Bertani broth? Is the ability of *E. coli* to grow at astounding speeds in rich laboratory media a feat that it is called upon to perform in nature? Answers may be forthcoming from studies such as those from Kurland's laboratory (56), which show that some individual isolates of *E. coli* do not initially grow rapidly in laboratory media and that this property may be acquired by selection of fast growing mutants.

Even in the large intestine, the environment rarely becomes nutritionally fallow because of the rich contents of the lumen and the materials secreted from the intestinal wall. In addition, some of the gut contents, especially those of vegetable origin, cannot be utilized by the host but are metabolized by resident microorganisms. Therefore, despite the oft-heard view, *E. coli* is probably not usually starving in the gut. The reason for its small numbers may have to be found elsewhere, perhaps in the inhibitory action of substances such as volatile fatty acids or sulfides. On the other hand, in the external environment, waters, and soils, *E. coli* is likely to starve most of the time and nutritional limitation is likely to play a more important role here than in the intestine.

Either inside or outside the vertebrate body, *E. coli* is under stress much of the time and is rescued from this condition by being grown in laboratory media. Studies on the stationary phase of growth (40, 46) will need to be complemented by work on slow-growing cultures, especially those whose growth is limited by naturally found inhibitors. The distinction between slow growth, chemical inhibition, and starvation may not be a strict one because we know already that the stationary phase is a dynamic state that involves much macromolecular turnover. However, information on the physiology of inhibited or very slow-growing cultures is scant. Besides asking if a given regulated system is "growth rate dependent" and "growth phase dependent," we may have to find whether it is "inhibition dependent."

When we think about ecological challenges, we must do so with appropriate modesty and recognize how easily a parochial outlook can trap us. The very term "enteric" to denote *E. coli* and *S. enterica* reveals the blased view that much of the evolution of these organisms has taken place in the gut. However, organisms such as *Klebsiella*, which resemble *E. coli* in the way they conduct their regulatory and metabolic activities, are in fact not intestinal organisms but are normally found in the soil (9). Is it a coincidence that in English the word "soil" has an appropriate double meaning?

Lastly, almost no bacteria, and certainly not *E. coli* and *S. enterica*, live in isolation outside the laboratory. There are worldwide populations whose integrity and genetic stability is awesome. The study of the population biology of bacteria has developed a firm molecular base, providing details about genetic sources of variation, rates of change, and structures of populations. This has been achieved both on a small scale, e.g., with populations of pathogens in individual hosts, and on a large scale, with populations that reach around the world (97, 103, 119). We know that the world populations of *S. enterica* and *E. coli* are clonal, making the importance of horizontal genetic exchange open to discussion. Against this background, genetic variation allows occasional favorable mutations to cause the replacement of entire populations (70).

The properties of naturally occurring microbial communities are not easy to study and may well require the development of new approaches. From such investigations, we expect that new views of how these organisms meet environmental challenges will arise. Ultimately, the study of all living things must include how they perform in their natural environment in association with other forms of life.

## CONCLUSION

In 1995, an editorial in Science (267:1575) commented that predictions made some 25 years previously regarding "Biology and the Future of Man" were largely fulfilled but that "the most revolutionary and unexpected findings were not predicted." We would be glad to do as well! As we stated at the beginning, our work as editors of the *Escherichia coli* and *Salmonella* book did not endow us with special powers of prophecy, but it does permit us to express our excitement for the future. In our opinion, *E. coli* and *S. enterica* will continue to play a central role in biological research. This is not because they are intrinsically more interesting than any other bacteria, as we believe that all are equally interesting. However, knowledge builds on knowledge, and it is here that these two species continue to have a large edge not only over other microorganisms but also, for some time to come, over all other forms of life. It is interesting in this connection that biotechnology, having made detours through other microorganisms, always seems to return to *E. coli* (108).

In our opinion, we know only a small part of how *E. coli* and *S. enterica* transact their business. Might knowledge of a cell, in fact, not be a receding horizon? It seems clear that what is left to know is far from trivial and that its pursuit is emphatically not a mopping-up operation. We believe that the "rest" of *E. coli* contains the answer to some of the majestic questions of cell biology. We find it particularly attractive that molecular microbiologists will join ecologists and computational scientists in probing the complex questions remaining. As they learn each other's languages and modes of thought and are ready to share technologies and ideas, they will fulfill the yearning for a deeper understanding of cellular functions.

Given all this, it should come as no surprise that a sequel to the second edition of *Escherichia coli* and *Salmonella* is already in early planning. As hinted at in the second edition, the sequel will hardly conform to the definition of an "edition," since it will become a continuously updated work, more comprehensive than its predecessors and more completely an account of the research effort of the entire community of *E. coli* and

*Salmonella* researchers. It will, of course, be digital, *EcoSal* will be its name, and it will occupy a website with extensive links to data-rich sites already in existence and those soon to come on line.

## WEBSITES DEVOTED TO *E. COLI*

There are several websites devoted to genetic and physiological data on *E. coli*: http://bmb.med.miami.edu/ecogene, http://genolist.pasteur.fr/colibri, http://www.genetics.wisc.edu, http://www.genome.ad.jp/kegg, http://www.cifn.unam.mx/Computational_Biology/regulondb, http://genprotec.mbl.edu, and http://ecocyc.PangeaSystems.com/ecocyc.html/ecocyc.

## APPENDIX

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