

MINIREVIEW

Just Toothpicks and Logic: How Some Labs Succeed at Solving Complex Problems

Howard A. Shuman*

*Department of Microbiology, College of Physicians & Surgeons, Columbia University,
New York, New York 10032*

When I was asked to write a biographical piece about Jon Beckwith, it was difficult to know exactly what to do. The details of Jon's career as a scientist are certainly of interest, and many people would be happy to either read or contribute testimonials to Jon's creativity and talent as a mentor. However, it seemed that this could instead be an opportunity to ask if there were any general principles that underlie the continuous success that the Beckwith lab has had in understanding complex problems, such as regulation of gene expression, membrane protein structure, protein secretion, cell division, and disulfide bond formation. Two themes may account for much of this consistency over time and over subject matter: the first is that there are several aspects of how problems are viewed and analyzed in what may be termed the "Beckwith style," and the second is related to the experimental tools of the bacterial geneticist and in particular the *lac* operon. These principles can be clearly seen from some early work on the genetics of *lac* regulation and the mechanism of conjugation as well as initial work on protein secretion in bacteria.

While some details of Jon's biography are relevant for this discussion, I refer readers to Jon's recent autobiography for an in-depth view of his experiences, particularly in the area of social and political activity, which are not covered here (3). Jon has been at the Department of Microbiology and Molecular Genetics at Harvard Medical School since 1965, when he was appointed an Assistant Professor (for a recent photograph, see Fig. 1). Prior to coming to Harvard, Jon had several postdoctoral experiences that undoubtedly contributed to the development of the Beckwith style and the adoption of the *lac* operon as a powerful tool.

ORIGINS

Despite an early interest in French literature, Jon decided to pursue a Ph.D. in biochemistry in the lab of Lowell Hager at the Harvard Chemistry Department. After studying microbial enzymes responsible for the synthesis of organic halides, Jon became aware of the work of François Jacob and Jacques Monod at the Pasteur Institute. Jacob and Monod, in collaboration with their mentor, Andre Lwoff, had shown that the

simple intestinal bacterium *Escherichia coli* and the bacteriophage λ , which uses it as a host, provided simple experimental systems for analyzing the logic and molecular basis of gene regulation. It is likely the simplicity of these experimental systems, plaque formation for λ and the plate phenotypes for the *lac* system of *E. coli*, that attracted Jon as a way to concentrate on the design and interpretation of experiments rather than focusing on the details of their execution.

When Jon wrote to Jacob asking if it would be possible to be a postdoctoral fellow in his lab, Jacob demurred, stating that it was not an opportune time and that it might be possible at some time in the future. Rebuffed by Jacob, Jon did the next best thing and worked at Berkeley and then Princeton with Art Pardee, who had just returned from working with Jacob and Monod. Pardee was, of course, together with Jacob and Monod, responsible for the eponymous "PaJaMo" experiments that provided the first genetic evidence for a diffusible repressor for the *lac* operon. The French group had recently described different classes of operator mutants; some, called O^c , resulted in constitutive *lac* gene expression and were postulated to define the site on the DNA where the repressor would act. Other operator mutants, called O^o , had a completely different phenotype; both the *lacZ* and *lacY* genes were expressed at very low levels, and consequently, O^o mutants could not grow on media where lactose is the sole carbon and energy source. The French were concentrating their efforts on the O^c mutants, so Pardee and Jon decided to explore the basis of the defect in the O^o mutants. What to do? The levels of β -galactosidase (product of the *lacZ* gene) and lactose permease (product of the *lacY* gene) had already been published, and there were also preliminary mapping data indicating that the O^o mutations mapped near the beginning of the *lacZ* gene. The prevailing and exciting view was that the O^o mutations defined a promoter, the site that controlled the frequency at which RNA polymerase would initiate transcription of the *lac* operon. Pardee and Jon decided to look for revertants of a particular O^o mutant, Lac2, to see if it was possible to regain *lac* operon expression. Perhaps the putative promoter could be partially active and express the *lac* operon to different extents. If so, it would provide strong evidence that the sites of the O^o mutants and the revertants defined the *lac* promoter. Generating Lac⁺ revertants was easy and mapping them was possible with the aid of Hfr and F' *lac* strains. Unfortunately, the revertants of Lac2 had nothing to do with the *lac* operon; they mapped elsewhere, far from the *lac* operon. In later work with

* Mailing address: Department of Microbiology, College of Physicians & Surgeons, Columbia University, 701 West 168th St., New York, NY 10032. Phone: (212) 305-6913. Fax: (212) 305-1468. E-mail: has7@columbia.edu.

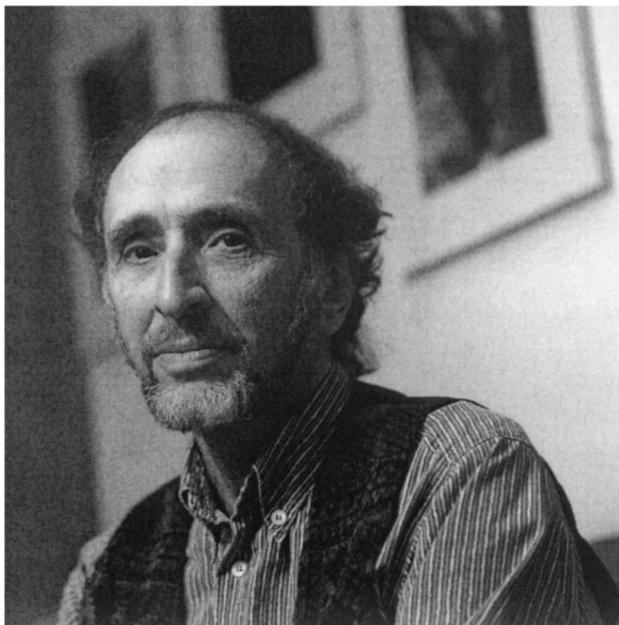


FIG. 1. Recent photograph of Jon Beckwith.

Sydney Brenner in Cambridge, England, Jon would show that Lac2 was really an ochre (TAA) stop codon early in the *lacZ* gene; the suppressors were later shown to correspond to mutated tRNA genes (8). The promoter hypothesis for Lac2 was wrong, but important information about the nature of the genetic code had been obtained. The selection and genetic analysis of Lac⁺ revertants had shed light on a fundamental aspect of biology.

Having discovered tRNA suppressors, Jon contacted Jacob again and asked if were possible to join the French group now that he had some experience with bacterial genetics. Apparently the time was not yet right, and Jon was rebuffed a second time. Having interacted with Brenner in Cambridge and Bill Hayes in London (in connection with Hfr strains and F' *lac*), Jon decided to spend time first with Hayes to learn about conjugation and then with Brenner to continue analysis of the Lac⁺ revertants.

While in Pardee's lab, Jon had learned that an α -galactoside, melibiose, was transported by the LacY permease even though it was not a substrate for β -galactosidase. Selection for growth on melibiose would permit isolation of Lac2 revertants that restored expression of the *lacY* gene without selection for expression of β -galactosidase. Mel⁺ Lac⁻ revertants of Lac2 were obtained, and mapping showed that some were linked to *lac* and others were elsewhere on the chromosome. One of the linked revertants turned out to be an in-frame 30-codon deletion removing the Lac2 stop mutation (4). This mutation, called *lacZM15*, formed the basis for the discovery of intragenic α -complementation of β -galactosidase and is used in every cloning experiment that takes advantage of the blue-white screen. The unlinked Lac2 revertants told a different story. These defined a gene called suppressor A, or SuA, which seemed to eliminate the polarity effects caused by the Lac2 stop mutation but did not restore β -galactosidase (5). Later work in John Richardson's lab showed that the SuA gene

encoded an essential transcription termination factor, Rho, needed for polarity (9, 16, 17a). Not a bad yield, considering that Lac2 had been completely "misunderstood" at the outset.

MOVING GENES

While in England in 1964, Jon finally got the go-ahead from Jacob on a postdoctoral position in his lab. Moving to Pasteur after being with Pardee, Brenner, and Hayes might have been anticlimactic, yet the lure of Paris was strong and the opportunity to join forces with Jacob, who had studied the mechanism of Hfr transfer with Elie Wollman, was irresistible. Arriving in Paris, Jon could also continue to collaborate with another American postdoc, Ethan Signer, with whom he had worked in Brenner's lab.

Jacob and his student, François Cuzin, had shown that a mutant form of F' *lac* that was not stable at a high temperature (42°C), F' *lac*⁺(Ts), could integrate into the *E. coli* chromosome at the restrictive temperature (11, 12). This could occur either by homologous recombination at the *lac* operon or in Δ *lac* strains, at a variety of sites all over the chromosome. Later, the events in the Δ *lac* strains would be shown to be the result of transposons on the F plasmid. Although Jacob and Cuzin had characterized the conjugal behavior of the F' *lac*⁺(Ts) strains and shown that they possessed different origins of transfer, they had not examined expression of the *lac* genes at the novel chromosomal locations. Jon decided to generate a new collection of F' *lac*⁺(Ts) insertions with the goal of characterizing positional effects on *lac* operon gene expression. An unexpected result however, diverted Jon's interest from studies on *lac* expression.

One of the new F' *lac*⁺(Ts) insertions also became auxotrophic; that is, the strain was no longer able to grow on minimal media due to a defect in the ability to synthesize a biosynthetic intermediate. Jon immediately realized that it should be possible to "direct" the insertion of F' *lac*⁺(Ts) to a particular gene, by looking for insertions with a specific phenotype. He was able to test this idea by taking advantage of the phage resistance phenotype of *tonB* mutants. The *tonB* gene encodes a component of the receptor for bacteriophage T1. Simultaneous selection for Lac⁺ and T1 resistance should yield strains in which F' *lac*⁺(Ts) inserted into the *tonB* gene. When such mutants were obtained, Jon and Ethan realized that because the *tonB* gene was adjacent to *att80*, it might be possible to direct the *lac* operon close enough to the lysogenic ϕ 80 phage DNA that upon phage induction, the ϕ 80 phage could carry the *lac* genes along with it. Isolation of several F' *lac*⁺(Ts) insertions in the *tonB* gene permitted isolation of different ϕ 80 phages that carried the *lac* genes (6). These ϕ 80*lac* phages represent the first time bacterial genes had been intentionally cloned on a variable-copy-number vector. Similar experiments carried out later by Karen Ippen and Jim Shapiro in Jon's lab at Harvard resulted in the isolation of λ *lac* phages (17). The isolation and biochemical manipulation of purified ϕ 80*lac* and λ *lac* DNA samples permitted Jon, Shapiro, and their collaborators to purify the *lac* operon, the first time a gene had been physically isolated and directly observed in the electron microscope (18). In addition to achieving a great deal of public attention, this work prompted Jon's reflection, in 1969, on the potential social misuses of genetics research. Needless to say,

these concerns have grown more concrete as genetic and stem cell technology have become more powerful.

MOVING PROTEINS

Following an intense period of discovery in the area of genetic regulation that included work on distinct functional sites in the *lac* promoter and the role of the positive transcription factor CAP in activating RNA polymerase, Jon was motivated to switch to areas that were less well studied. In the mid-1970s the question of how specific proteins were targeted to particular compartments within cells was just being approached. The groundbreaking work of Gunther Blobel and Bernhard Dobberstein suggested that specific signal sequences were used to direct secreted proteins out of the cytosol (7). The contribution of the signal sequences and the nature of the secretion machinery were completely unknown at that time. Even so, the “signal hypothesis” was novel and fascinating, and Jon realized that it would be possible to bring the “awesome power of bacterial genetics” to bear on testing Blobel and Dobberstein’s hypothesis. Also, the question of whether there were signal sequences in bacteria and, if so, whether they were required for secretion could be answered with *lac* gene fusions.

The capacity to fuse any *E. coli* gene to *lacZ* was a natural outgrowth of the earlier work on transposition of *lac*-transducing phages mentioned above. Jon’s student Malcolm Casadaban realized the potential to expand and facilitate the construction of *lacZ* gene fusions with the transposable bacteriophage Mu (10). In addition, Casadaban’s work made it possible to produce translational *lacZ* fusions that would result in hybrid proteins with β -galactosidase. A happy coincidence of Casadaban’s development of a suite of in vivo tools for making fusions to virtually any *E. coli* gene in a matter of days and the arrival of two postdoctoral fellows, Tom Silhavy and Phil Bassford, who were anxious to apply these tools to genes encoding secreted and membrane proteins enabled rapid progress in the construction and analysis of *lacZ* fusions to the extracellular components of the maltose transporter. These include a periplasmic maltose binding protein (MBP); an outer membrane protein porin (LamB, λ receptor), and an inner membrane protein, MalF. (Adoption of the maltose transport system as a target for producing *lacZ* fusions had another felicitous side product. Essentially all of the genetics of the maltose system had been done in the labs of Maxime Schwartz and Maurice Hofnung, both at the Institut Pasteur. In the 1960s, Schwartz had been a doctoral student of Monod and Hofnung a student of Jacob. Thus, not only were the *lac* and *mal* genes fused, but so were the labs in Boston and Paris.) Thus, in a relatively short time, *lacZ* had been fused to genes that encode proteins in all three extracytoplasmic locations. Were there signal sequences in these genes? If so, were the signal sequences sufficient to localize the β -galactosidase hybrids to the extracytoplasmic location? There were quite a few surprises; expression of some of the monster β -galactosidase hybrids resulted in funny-looking colonies that did not grow well or lysed (2). It is very likely that most mentors would have encouraged their young postdocs to “put away” the uncharacterized funny-looking colonies and focus on the ones that behaved normally. Not in Jon’s lab; in his work with Silhavy and Bassford, questions were asked. Why are some of the hybrids

toxic? What happens if nontoxic suppressors are selected for? Are all of the suppressors linked to the fusions? Where are the unlinked ones? The results came fast and furious. The funny-looking fused MBP-*LacZ* gene had most of the coding sequence for MBP, and the hybrid protein was found in the inner membrane, not the periplasm. The same result was obtained with the funny-looking LamB-*LacZ* hybrid (19). As the strains accumulated these monsters, they died, and as they died, the export of other secreted proteins was blocked—higher-molecular-weight precursors of alkaline phosphatase, OmpA, and many other proteins were observed for the first time as the monsters blocked the cells’ ability to secrete all of the other proteins that normally are destined for the periplasm and outer membrane. It was easy to get mutants that still produced MBP-*LacZ* or LamB-*LacZ* hybrids that were not toxic. Genetic mapping put the mutations at the 5’ end of the gene. When the 5’ regions of the wild-type and mutated genes were sequenced (a novel concept at the time), they revealed nucleotides encoding a signal sequence in the wild-type copy of the gene that was not found in the mature polypeptide; in the nontoxic mutants there were alterations that either deleted key hydrophobic amino acids or introduced charged residues in the midst of the hydrophobic regions. Once it was shown that these mutations also had effects on the secretion of the unfused MBP and LamB proteins, the signal hypothesis of Blobel and Dobberstein became dogma (14).

The availability of the hybrids and the signal sequence-defective genes also made it possible to identify components of the secretion machinery itself. The secretion components SecA, SecB, and PrlA (SecY) were all originally identified in Jon’s and Silhavy’s labs as a result of simple genetic screens done with the monsters or their progeny. Many of these selections and cute tricks relied on intuition in addition to logic, but all used toothpicks and agar plates but not a single unit of a restriction enzyme or a plasmid. Indeed, it is likely that the same genes on high-copy-number plasmids would never have produced the phenomena that prompted the development of the successful genetic screens and selections.

MOVING ELECTRONS

One of the last peculiarities of the collection of *mal-lacZ* fusions was a particular *malF-lacZ* fusion that, in contrast to all the other *malF-lacZ* fusions, had very low levels of β -galactosidase activity (15). Again, it would have been simple to dismiss its low activity out of hand for any one of a number of trivial reasons. Yet Jon persevered and found out that that the fusion joint between the MalF polypeptide and β -galactosidase was in an extracytoplasmic loop of the MalF polypeptide. Why should this make a difference in β -galactosidase activity? Is the MalF part of the hybrid forcing the β -galactosidase part out to the periplasm? If so, why would it stay there? Shouldn’t it be able to fold properly in the cytosol rather than be dragged into the periplasm? There was really only one solution for answering these questions: look for mutations that restore higher levels of β -galactosidase to the inactive MalF-*LacZ* hybrid. When these mutants were obtained by Karen McGovern and Jim Bardwell, the mutations mapped to a new gene. This new gene, christened *dsbA*, encodes a product resembling a family of enzymes called protein disulfide bond isomerases (1). Null mutations in

dsbA permitted the MalF-LacZ hybrid to fold in the cytosol and exhibit just as much activity as all the other naturally cytosolic MalF-LacZ hybrids. Apparently, the presence of DsbA permits the formation of disulfide bonds in the β -galactosidase part of the one MalF-LacZ hybrid that in turn cause it to fold and be trapped in a form that remains transmembrane and inactive. In many bacterial species, DsbA is required for the presence of a variety of extracellular organelle components and toxins. Rather than focus on the particularities of the MalF-LacZ hybrid, Jon's attention turned to the pathways of electrons that are used in disulfide bond formation (13). Where do they originate? How do the electrons get to DsbA? What are the connections between electron flow, disulfide bond formation, and protein folding? These are questions still in formation and still being answered—with toothpicks and logic.

ACKNOWLEDGMENTS

I could not have written this piece without the help of many people. First, Jon was enormously generous with his time and helpful in discussing and clarifying several aspects of the early work on directed transposition of the *lac* genes. In addition, Stanley Brown, Susan Gottesman, and Tom Silhavy were kind enough to read drafts of the manuscript and offer constructive suggestions for improvement. Several former Beckwith alumni and alumnae were generous in sharing anecdotes and impressions of their time in Jon's lab. Finally, Tom believes the phrase "just toothpicks and logic" derives from an apocryphal statement by Sydney Brenner.

REFERENCES

1. Bardwell, J. C., K. McGovern, and J. Beckwith. 1991. Identification of a protein required for disulfide bond formation *in vivo*. *Cell* **67**:581–589.
2. Bassford, P. J., Jr., T. J. Silhavy, and J. R. Beckwith. 1979. Use of gene fusion to study secretion of maltose-binding protein into *Escherichia coli* periplasm. *J. Bacteriol.* **139**:19–31.
3. Beckwith, J. 2002. Making genes, making waves: a social activist in science. Harvard University Press, Cambridge, Mass.
4. Beckwith, J. R. 1964. A deletion analysis of the *lac* operator region in *E. coli*. *J. Mol. Biol.* **8**:427–430.
5. Beckwith, J. R. 1964. Restoration of operon activity by suppressors. *Biochim. Biophys. Acta* **76**:162–164.
6. Beckwith, J. R., and E. R. Signer. 1966. Transposition of the *lac* region of *Escherichia coli*. I. Inversion of the *lac* operon and transduction of *lac* by ϕ 80. *J. Mol. Biol.* **19**:254–265.
7. Blobel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.* **67**:835–851.
8. Brenner, S., and J. R. Beckwith. 1965. Ochre mutants, a new class of suppressible nonsense mutants. *J. Mol. Biol.* **13**:629–637.
9. Brown, S., B. Albrechtsen, S. Pedersen, and P. Klemm. 1982. Localization and regulation of the structural gene for transcription-termination factor rho of *Escherichia coli*. *J. Mol. Biol.* **162**:283–298.
10. Casadaban, M. J. 1975. Fusion of the *Escherichia coli lac* genes to the *ara* promoter: a general technique using bacteriophage Mu-1 insertions. *Proc. Natl. Acad. Sci. USA* **72**:809–813.
11. Cuzin, F., and F. Jacob. 1967. Mutations of the F episome of *Escherichia coli* K 12. II. Mutants with thermosensitive replication. *Ann. Inst. Pasteur (Paris)* **112**:397–418. (In French.)
12. Cuzin, F., and F. Jacob. 1967. Stable association of 2 different F episomes in a clone of *Escherichia coli*. *Ann. Inst. Pasteur (Paris)* **113**:145–155. (In French.)
13. Debarbieux, L., and J. Beckwith. 1999. Electron avenue: pathways of disulfide bond formation and isomerization. *Cell* **99**:117–119.
14. Emr, S. D., M. Schwartz, and T. J. Silhavy. 1978. Mutations altering the cellular localization of the phage lambda receptor, an *Escherichia coli* outer membrane protein. *Proc. Natl. Acad. Sci. USA* **75**:5802–5806.
15. Froshauer, S., G. N. Green, D. Boyd, K. McGovern, and J. Beckwith. 1988. Genetic analysis of the membrane insertion and topology of MalF, a cytoplasmic membrane protein of *Escherichia coli*. *J. Mol. Biol.* **200**:501–511.
16. Guarente, L. P., and J. Beckwith. 1978. Mutant RNA polymerase of *Escherichia coli* terminates transcription in strains making defective rho factor. *Proc. Natl. Acad. Sci. USA* **75**:294–297.
17. Ippen, K., J. A. Shapiro, and J. R. Beckwith. 1971. Transposition of the *lac* region to the *gal* region of the *Escherichia coli* chromosome: isolation of *Malc* transducing bacteriophages. *J. Bacteriol.* **108**:5–9.
- 17a. Richardson, J. P., C. Grimley, and C. Lowery. 1975. Transcription termination factor Rho activity is altered in *Escherichia coli* with *SuA* gene mutation. *Proc. Natl. Acad. Sci. USA* **72**:1725–1728.
18. Shapiro, J., L. Machattie, L. Eron, G. Ihler, K. Ippen, and J. Beckwith. 1969. Isolation of pure *lac* operon DNA. *Nature* **224**:768–774.
19. Silhavy, T. J., H. A. Shuman, J. Beckwith, and M. Schwartz. 1977. Use of gene fusions to study outer membrane protein localization in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **74**:5411–5415.