Further information on Research Plan:
Studies on the Mechanism of Transformation in B. subtilis

This material is submitted in response to a request to furnish the finer brushstrokes that will paint in the details ... so that the [study section] may find themselves better able to evaluate the finalized picture."

In my covering letter, I offered some of the reasons that make this a difficult task, if the research plan is expected to be a reliable chart of future developments. Attachment (1) illustrates the role of unexpected findings in changing the detailed plan on a short time scale, and knowing that this is the main fruit of our effort makes the construction of very elaborate plans seem an exercise in futility. In addition, we face the special problems of satisfying foreseeable regulatory restrictions that complicate efforts to examine some of the most telling scientific issues.

The field of genetic transformation also has a long history. Fortunately, Microbiology-1976 has appeared since the original proposal was submitted. It includes extensive reviews by Frank Young, David Dubnau and others, that cover most of the issues of concern for the present status of the mechanism of transformation. I hope it is legitimate for me to refer to that source rather than repeat a great deal of detail that is already in print.

Dubnau summarizes the stages of transformation as

1) binding
2) double-strand fragmentation of DNA (on the cell surface)
3) single-strand formation
4) interaction with recipient DNA
5) postsynaptic events (resolution of the heteroduplex DNA) expression, etc.

As mentioned earlier, it was my plan to reexamine each of the classical experiments that led to this model (some of which were done here: Bodmer & Ganesan '64-66) with the use of HOMOGENEOUS populations of genetically (and isotopically) marked DNA such as is now accessible through the amplification of inserted DNA segments in plasmid vectors. Dubnau furnishes an apt quotation to illustrate the motive: "Our knowledge of the impact of DNA structure and chemistry on binding is rudimentary." There are many confusing and conflicting claims about the influence of single-strandedness, nicks and other structural modifications of the DNA on binding, and thereafter in the whole process.

Even such a simple issue as the role of DNA size on the efficiency of transformation cannot be readily rationalized; though it has long been known that larger molecules (about 10 megadaltons) are most effective. Even here, lacking homogeneous samples, it is difficult to separate issues of size from freedom from damage, and freedom from competition from DNA lacking the specific markers.

Since DNA bound to the cell can be sheared off, it would also be desirable to have homogenous material to look for the specificity of the binding site on the DNA: is it preferentially one end; are there preferred sites interstitially, and so on.

The basic theme of all these experiments is simply to trace the fate of marked donor DNA through the 5 stages summarized above. The use of homogenous (amplified DNA) with well defined termini offers several advantages: high specific biological activity; predetermination of the relevant input segment, e.g., so that it can be recovered later by re-segmenting the target DNA with the same restriction enzymes; definition and control of the other input DNA species present. Current experiments use fragmented DNA of variable size, extent and terminations, and generally highly mixed with fragments of the entire source genome. With such heterogeneous DNA, some tests are qualitatively unfeasible; others, e.g. precise measures of the timing and variability of fragmentation, complex formation, etc., could not be made with very high precision and interesting anomalies would then be obscured.

For example, some of our recent work (Harris-Warwick et al., 1975) used partially purified EcoRI-segments. These showed graded efficiency of marker transfer that we speculated came from 'edge-effects', namely that markers near the boundary of the segment were more likely to be eroded at an early stage of transformation than those at the center. This is a simple, perhaps even intuitively obvious expectation. However, there were still some uncomfortable discrepancies between this view and the linkage statistics. Most desired would be a series of amplified segments, embracing the same general region, but tailored at the ends for a direct measure of the effect of the geography of a marker on its acceptance in the chromosome. There should be no difficulty in finding, and we have some strong hints already of mutations in the restriction-target site that will give us segments of different sizes as material for such experiments. Conversely, we can seek deletions near the edges that will bring critical markers closer to an edge.

In older work, many people may have noticed anomalies in the frequency of co-transfer (linkage) of a pair of markers as a function of DNA concentration [sic] and of size. These might be explained by a peculiar kinetics of competition between the larger (2-marker-embracing) fragments and smaller ones, both from the same region and from other parts of the chromosome. In the past, we could not reliably test our primitive speculations put forward to explain such anomalies; we certainly were in no position to make observations that would critically challenge the consensual picture.

It may illustrate what we are looking for to recall how the advent of Hfr strains of E. coli, and the careful kinetic studies of E. coli conjugation by Jacob & Wollman were instrumental in uncovering what was really going in in E. coli crossing. Kinetics of that level of precision has not really been possible until now with inhomogenous transforming DNA.

The basic experimental methods are already well-established, in other laboratories and in our own. Besides the conventional biochemical-genetic marker mapping methods, DNA used in uptake and integration studies would be double labelled (a la Bodmer & Ganesan, for example) to permit pycnographic separation of mixed complexes of donor and recipient DNA. Now we have the additional advantage of restriction-enzyme-segmentation of the target DNA, fractionation by gel-electrophoresis, and heteroduplex analysis by electronmicrography, or with c-RNA staining (a la Sothern ). {The latter is particularly pertinent in dealing with complexes involving heterologous segments, as we have studied in B. subtilis/ globigii crosses; and which arise in confrontations of recombinant plasmids with the recipient DNA.}

With honogeneous and amplified DNA we also expect to be in a far better position to study the specificity of competition at every stage of the process -- being able to make well formed mixtures of known inputs. Likewise, the search for and categorization of mutants that influence the efficiency of transformation, either with regard to the donor DNA or the recipient, will be greatly facilitated. A number of recombination-deficient mutants are known, mostly concerned with uptake or polymerase-defects; much less is known especially in B. subtilis about variations in the donor DNA arising by mutation.

There remains the task of getting appropriate material for such studies. Our reported finding (soon going to press) about the expression of some B. subtilis genes in E. coli opened the door to the use of E. coli plasmids as vectors, and was our initial plan. However, for many reasons -- including biohazard considerations especially in the current regulatory climate -- we would prefer a system that allowed us to amplify subtilis DNA in B. subtilis cells. We realize there may soon be many alternative possibilities for this; but are relieved that there is one very likely avenue by exploiting the growth of Staphylococcus plasmids in B. subtilis. These plasmids are technically [e.g. size, number of restriction sites, selectible markers] superior to any other that we know of right now in Bacillus. Yes, we have heard 'rumors' about E. coli plasmids also being transferable to subtilis, but would be in some dilemma if we had to stake this proposal on unpublished information from other laboratories.

We are just now in the process of trying to fulfill the promise that the staph plasmids offer as cloning vehicles, which entails the usual experiments of ligating sticky-ended marked segments to restriction-enzyme-segmented plasmid DNA. It is some advantage that we already have phi-3-t segments of known efficacy for the thy+ marker, and can also try the subtilis segments that have been cloned and found to effect transformation both in E. coli and and in subtilis.

In addition, we are just starting some experiments on the transmissibility of staph plasmids to B. subtilis in mixed culture, viz., by some conjugal mechanism. This could be an operational convenience; perhaps more important today, it would also throw some light on the promiscuity of bacterial gene exchange under conditions even less intrusive than the extraction of DNA for transfection.

These experiments were motivated by Davies' speculation that antibiotic-resistance factors in common pathogens ultimately originated in soil actinomycetes which are the source of antibiotics, and which have evolved adaptations to cope with them in nature. If this can be substantiated, we are likely to find many other instances of 'promiscuous' gene exchange as a natural process. Indeed, several other workers are energetically seeking other wide exchanges of plasmids with B. subtilis. It is quite likely that the present staph system will not be the ultimately ideal one, and we are prepared to shift gear in the light of developing new information.

Finally, we have in mind that the staph plasmids appear to be unstable in B. subtilis -- an important biological and methodological issue. We will inevitably be preoccupied with what is behind stability. What will doubtless happen is that, incidentally to other work, changes of stability pattern will be noted: and if this occurs in an experimentally advantageous context, we will try to understand what genetic and physiological factors influence the fitness of the exotic plasmid. In particular, insertions of additional segments may have paradoxical effects: from previous experience, larger plasmids will have some trouble propagating. But in some cases the augmentation of the plasmid with segments of the host genome may facilitate the durability of the plasmid. The fact that, so far as we know, there is no detectable homology of DNA sequences in the plasmid and in the chromosome is actually highly advantageous for further analysis. (We are having some trouble in tracking phi-3-t on this account.)