PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Genetic Engineering of Bacilli [and Discussion]

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Genetic engineering of bacilli

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Bacillus subtilis has emerged as a major cloning system. In this paper we analyse the vectors, vehicles and strategy for cloning. Solutions to current problems in genetic engineering including the development of standard fermentation strains are proposed.

Introduction

The science of microbiology is rooted in applied fields such as industrial and medical microbiology as exemplified by the work of Pasteur. Krause (1981) emphasized 'Just a little more than a century ago, through research that was by turns imaginative, practical, and resourceful, Pasteur proved that decay, petrification and fermentation were the work of microbes. And he recognized the similarity between infectious disease in plants and those in animals and man.' From these seminal observations, microbiology developed as both a fundamental and an applied field of research. It was the advent of recombinant DNA technology, however, that enabled scientists to abandon the random approach of strain variation through mutation and selection and to design unique organisms for gene expression by manipulation and intervention in the hereditary code of the living cell. In our haste to apply this technology to industrial processes, we must not only acknowledge our vast debt to basic research and repress our hubris but continue to nourish that very fundamental vein of inquiry that serves to sustain scientific creativity. Thus, 'We stand as pygmies on the shoulders of giants sifting our observations through the grid of our prejudice in an attempt to approximate truth not merely to sacrifice it on the altar of our ego but to serve mankind' (Young 1978). Furthermore, it is important to reflect on the history of the recombinant DNA debate, to be ever mindful of the intense public concern that surrounded the initial phase of this debate (Swazey *et al.* 1978), and to remain sensitive to the concerns of the public as we move into a new era of interventive industrial microbiology.

Bacilli as a model system

The years of basic research in the *Escherichia coli* model system converged through four major advances to form the foundation for the field of recombinant DNA (table 1). The proposed experiment of Berg to clone SV40 viral genome within bacteriophage λ precipitated the moratorium on recombinant DNA research and culminated in the Asilomar guidelines and the first detailed guidelines for research with recombinant DNA molecules promulgated in 1976. Appendix A of these guidelines on the applicability of the *Bacillus subtilis* system for studies in rDNA concluded 'Based on its promise, it seems appropriate and not chauvinistic to urge the development of this system' (Young 1976). Since the formulation of that appendix, a number of cloning vectors and vehicles have been developed, significant new basic information

has been amassed, and a number of applications to the cloning of bacterial and eukaryotic genes have been presented.

Three major factors led to the development of the *Bacillus* system. First, the organism is a non-pathogenic soil organism (Young & Wilson 1978) that can be readily mutagenized to produce asporogenic strains. One of these isolated by S. Mottice has been recently certified by

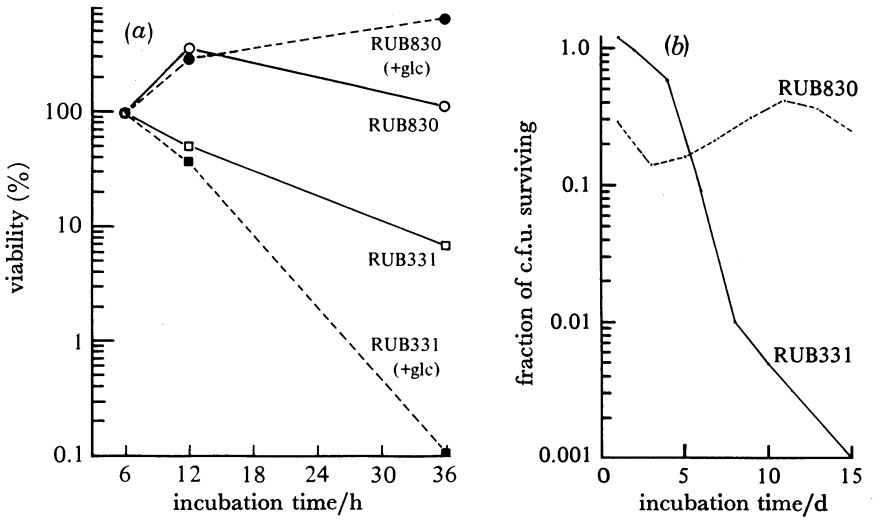

FIGURE 1. (*a*) Viability of *B. subtilis* strains RUB331 and RUB830. Cells were grown in M broth (0.1 mM Fe $(NO_3)_3$) with and without the addition of glucose (5 g l^{-1}). The viability was determined by plating samples at various times. For each culture, viabilities were normalized to the values at 6 h (= 100%). The 6 h values were: for strain RUB331 (□), 5.1×10^7; for RUB331 in glucose (■), 2.6×10^7; for strain RUB830 (○), 1.3×10^8; for RUB830 in glucose (●), 3.0×10^7.

(*b*) Fraction of colony-forming units (c.f.u.) of *B. subtilis* strains RUB830 (– – –) and RUB331 (——) surviving in soil culture. Strains RUB830 and RUB331 were grown in soil, and population sizes were monitored by the methods of Graham & Istock (1978): the starting inoculum sizes were 4.56×10^6 c.f.u. g^{-1} for RUB830 and 4.28×10^5 c.f.u. g^{-1} for RUB331. Each point is the mean population size of at least three replicate cultures divided by the starting inoculum size. The fraction of the RUB331 starting inoculum recovered as spores (i.e. resistant to heating at 80 °C for 15–20 min) was 10^{-5} on day 2, 8×10^{-4} on day 4, 2×10^{-4} on day 6, 5×10^{-5} on day 10, and 10^{-3} on day 15. These values are not statistically distinguishable from one another. Further testing of colonies arising from heat-shocked samples indicated that these spores most probably arose from leakiness rather than from reversion of the mutation.

TABLE 1. MAJOR ADVANCES LEADING TO rDNA TECHNOLOGY

discovery	use
site-specific enzymes	break DNA at unique sites
ligase	joins DNA fragments
plasmids	contain foreign DNA segments
transformation of *E. coli*	propagate or grow plasmids after transformation

the Recombinant DNA Advisory Committee permitting the cloning of a vast array of genes in *B. subtilis* (Young 1980). This strain is readily inactivated both in media and in soil, as shown in figure 1 (Young 1980). The loss of viability is probably related to the activity of endogenous autolytic enzymes (Brown & Young 1970). The organism can therefore be readily manipulated under safe laboratory conditions. Second, *B. subtilis* has a well defined genetic map (Young & Wilson 1972, 1975; Henner & Hoch 1980; Piggot & Coote 1976) that has 340 mapped genes (Dean & Dooly 1981). The organism is readily manipulated by DNA-mediated transformation

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(Spizizen 1958), generalized transduction by PBS-1 (Takahashi 1961), SP10 (Thorne 1968), and SPP1 (Yasbin *et al.* 1975) and by specialized transduction (Zahler *et al.* 1977). More recently, Chang & Cohen (1979) demonstrated that protoplasts of non-competent strains can be transformed by plasmid DNA, thus avoiding the normal conversion of double-stranded DNA to a single-stranded DNA during the natural transformation process. Taken collectively, these results ensure the rapid development of procedures to introduce plasmids into a variety of bacilli. Finally, the transfection of the stable L-form (Young *et al.* 1970) by bacteriophage ϕ25 (White & Streips 1982) provides a model for the use of this stable and well characterized wall-deficient mutant (Gilpin *et al.* 1973) in both genetic and fermentation studies. Third, bacilli have been extensively used in fermentation processes for the production of both commercial enzymes and antibiotics (Erickson 1976; Priest 1977). Of the more than 40 products produced by bacilli, the α-amylases and the proteases are currently the most significant enzymes.

Plasmid cloning vectors in bacilli

The initial studies focused on chimeric plasmids composed of either *E. coli* plasmids such as pMB9 or pBR322 and a cloned segment of bacterial or bacteriophage DNA. By using the gene encoding the thymidylate synthetase from bacteriophage ϕ3T, a single copy of a cloned gene could be integrated into the chromosome of *B. subtilis* after the propagation of the chimeric plasmid in *E. coli* (Duncan *et al.* 1978). The subsequent discovery of *Bacillus pumilus* plasmids that can propagate in *B. subtilis* (Lovett *et al.* 1976) followed by the extremely important discovery that plasmids from *Staphylococcus aureus* can propagate in *B. subtilis* led to an explosive development of both intragenic and intergenic (shuttle) plasmids, as reviewed by Dean & Dooley (1981). In principle, these shuttle plasmids permit the exchange of genes between broadly diverse species such as *E. coli* and *B. subtilis*. If there is no homology between the chromosome of *B. subtilis* and the chimeric plasmid, the plasmid can be readily propagated in *B. subtilis* as a plasmid. If homology exists, however, the plasmid is incorporated into the chromosome as in pCD4 (Duncan *et al.* 1978). The integration can be readily overcome by the introduction of the *rec*E mutation (Young 1980). Occasionally, foreign genes that do not have any apparent homology can be integrated into the chromosome of *B. subtilis*; this is exemplified by the gene encoding thymidylate synthetase from *E. coli* as shown in table 2 (Rubin *et al.* 1980). Cleavage of this chimeric plasmid by a site-specific endonuclease results in a marked reduction in the frequency of transformation. No transformation occurs with chromosomal DNA from *E. coli*.

Bacteriophage vectors

A number of bacteriophages have been studied as vectors. The most promising vectors are shown in table 3. Bacteriophage ρ11 and ϕ3T are readily used for cloning. However, these are extremely large bacteriophages and it is difficult to reisolate the fragment after the initial cloning event. Bacteriophage SP02 has been extensively characterized physically. However, it has not been possible to insert any genes into the deletion mutants. The recent work of Heilmann & Reeves (1982) is most intriguing. They have isolated a vector SPP1v* that exploits the well analysed bacteriophage promoters and has no *Bam*H1 site. Through the introduction of linkers for *Bam*H1 and the use of either *Bcl*I or *Bgl*II, it is possible to exploit the *Bam*/*Bgl*II fusion technique developed by Young & Wilson (1978). Through this methodology, only recombinants

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

survive (figure 2). The genes were then re-excised with other nucleases such as *Xma*III. Because the system has been developed to clone pUB110 and pBR322, it is possible to study autonomous replication in either *B. subtilis* or *E. coli*.

TABLE 2. Thy$^+$ TRANSFORMING ACTIVITY OF CHIMERIC PLASMIDS IN *B. SUBTILIS*

donor DNA	transformants per 10^8 cells: no enzyme treatment	*Bam*H1
B. subtilis 168 chromosomal	3.0×10^5	n.d.†
E. coli K-12 chromosomal	< 10	n.d.
pER2 (*E. coli thy*A)	6.0×10^4	4.5×10^2

1 μg of DNA was used to transform *B. subtilis* strain RUB830. pER2 was constructed by cloning the Thy$^+$ trait from *E. coli* into the *Eco*R1 site of pBR322 (Rubin *et al.* 1980). Transforming activity was determined as the number of transformants obtained with 1 μg DNA used as a *B. subtilis* recipient strain the *Thy*A-*Thy*B mutant RUB830.

† n.d., not determined.

TABLE 3. BACTERIOPHAGE VECTORS DEVELOPED FOR CLONING IN *B. SUBTILIS*

bacteriophage	molecular mass/MDa	site of integration	phenotype	reference
ρ11	80	*att*ρ11	Leu$^+$His$^+$Lys$^+$	Kawamura *et al.* (1979)
ϕ3T	80	*att*ϕ3T	α-amylase	Yoneda *et al.* (1979)
ϕ105	24	*att*ϕ105	plasmids encoding resistance	Marrero & Lovett (1980)
SP02	24	*att*SP02	none	Yoneda *et al.* (1979)
SPP1	27	none	plasmid pUB110	Heilmann & Reeves (1982)

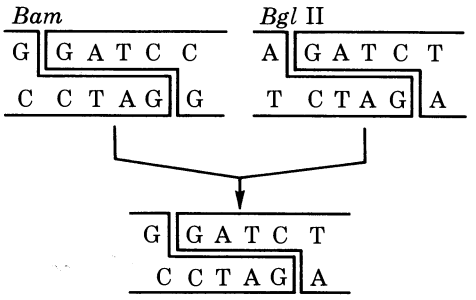

FIGURE 2. *Bam*/*Bgl* fusion. Incubation of DNA with site-specific endonucleases *Bgl*II and *Bam*H1 in the presence of ligase produces a unique site.

NOVEL EXPLOITATION OF THE *B. SUBTILIS* SYSTEM

In the fermentation industry, it is important to develop a limited number of strains that can be widely used for the production of a variety of products. One approach is the development of cloning systems that combine both chromosomal and vector-encoded genes. For example, Yoneda *et al.* (1979) cloned the α-amylase from *B. amyloliquefaciens* on to bacteriophage ϕ3T. When this bacteriophage is introduced into an amylase-negative strain (table 4), there is an increase from 0.87 to 63.8 units ml^{-1} of α-amylase, compared with the normal production of 11.0 units ml^{-1} found in the non-engineered strain. When the engineered strain also carries the *pap*M locus, however, the level of production is markedly enhanced. Thus, increased levels of a variety of products could be produced by fusing the α-amylase gene to a number of cloned products.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Another approach of major significance involves the development of shuttle vectors encoded within λ phage charon. As noted by Ferrari *et al.* (1981), the charon 4A phages can be used to clone the chromosome of *B. subtilis*. If a non-essential fragment of a temperate bacteriophage or a fragment of the shuttle vector were inserted into the cloning vector, the entire vector and cloned fragment should be integrated at the attachment site of the temperate bacteriophage or

TABLE 4. PRODUCTION OF α-AMYLASE BY VARIOUS *B. SUBTILIS* STRAINS CARRYING CLONED α-AMYLASE GENE

strain	relevant genotype	additional traits	total α-amylase activity
6160	*amy*E$^+$, *amy*R1	Amy$^+$	11.00
RUB210	*amy*E07, *amy*R1	Amy$^-$	0.87
RUB211	*amy*E07, *amy*R1	ϕ3T-Amy$^+$	63.80
RUB212	*amy*E07, *amy*R1	*pap*M, Amy$^-$	0.56
RUB213	*amy*E07, *amy*R1	*pap*M, *Amy*$^-$, ϕ3T-Amy$^+$	1516.10

TABLE 5. POTENTIAL PROBLEMS IN THE *B. SUBTILIS* MODEL SYSTEM

problem	Attempted solutions
expression	synthesis of appropriate signals cloning adjacent to natural promoter and ribosomal binding sites
variability of cloned fragment	isolation of recombination-deficient mutants selective pressure integration of multiple chromosomal copies
degradation of product	isolation of protease deficient mutants isolation of asporogenic strains excretion of product extracellularly

within the shuttle vector. In the latter case, the host recombination system should theoretically serve to integrate the incoming DNA and thereby broaden the host range. The observation that transformation can occur with intact λ bacteriophage (Ferrari *et al.* 1981) provides an additional convenience to this approach.

POTENTIAL PROBLEMS IN THE *B. SUBTILIS* SYSTEM

The vectors and host modifications described above, coupled with the development of gene libraries of the chromosome of *B. subtilis* (Rapoport *et al.* 1979; Hutchinson & Halvorson 1980; Ferrari *et al.* 1981) have provided the tools that are required for the expression of bacterial genes. Three potential problems should be addressed (table 5). Unlike the case in *E. coli*, foreign genes are not readily expressed in *B. subtilis*. For example, although the gene encoding thymidylate synthetase from *E. coli* could be expressed in *B. subtilis*, the gene encoding β-lactamase on pBR322 was not expressed (Ruben *et al.* 1980). The observations of differences in initiation of transcription between *E. coli* promoters and *B. subtilis* promotors (Lee *et al.* 1980), coupled with the changes observed in transcription of *B. subtilis* genes during sporulation (Ollington *et al.* 1981) suggest that the expression of foreign genes will require a careful construction of promoters recognized by the *B. subtilis* transcription systems. Such an approach was used by Williams *et al.* (1981) to express the mouse dihydrofolate reductase gene by using a promoter cloned from bacteriophage SP02. Alternatively, the foreign gene can be introduced within a *B. subtilis* gene as in the cloning of hepatitis B core antigen and the major antigen of

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

foot and mouth disease virus (Hardy *et al.* 1981). In addition, there may be similarities among *Bacillus* polymerases that will permit direct expression of a foreign gene from one *Bacillus* species to another. For example, the δ subunit of the RNA polymerases from *B. subtilis* and *Bacillus thuringiensis* appeared to be interchangeable (Achberger *et al.* 1982).

The variability of the cloned plasmid may present a more challenging problem. In many laboratories, including our own, deletions of plasmids have been observed during transformation (Uhlen *et al.* 1981; Grandi *et al.* 1981; Primose & Ehrlich 1981). The loss is related to two major factors: segregational instability and structural instability (Primose & Ehrlich 1981). While the exact mechanism of deletions in the latter case is not known, it does not appear to be related to the *rec*E4 function nor to base homology between the chromosome and plasmid. To overcome this problem, it may be necessary to devise selective pressure techniques that can be used in large-scale fermentations or to insert the foreign fragment in many locations in the chromosome by using lysogenic bacteriophages as vectors or to insert genes adjacent to linkers and promoters, constructed *in vitro*.

The problem of proteases remains a vexing one. By using the well characterized genetic map of *B. subtilis*, it may be possible to inactivate the genes related to protease production directly or indirectly through the selection of appropriate asporogenic mutants. Additionally, protection may be obtained by fusing the desired protein to an exoenzyme or sequestering it in a cell in the fashion analogous to the production of the crystalline protein in *B. thuringiensis*. The comparison of production of the protein in *B. thuringiensis* and in *E. coli* (Schnepf & Whiteley 1981) may elucidate the potential of such an approach.

Concluding remarks

The explosion of genetic and physiological studies in *B. subtilis* since the discovery of transformation by Spizizen in 1958 has provided the basic foundation for the studies in recombinant DNA. The extensive exploitation of this model since the development of the N.I.H. guidelines has been remarkable in view of the impediment to progress with this model system resulting from the promulgation of guidelines and regulations in many countries around the world. Currently, the fundamental knowledge has been developed for the widespread use of this organism for the production of cloned products. In our rush to apply technology to industrial processes, we must not forsake the continued support of basic research that enabled this goal to be attained.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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Discussion

A. R. Thomson (*Biochemistry Group, A.E.R.E. Harwell, U.K.*). The recovery and purification of products from fermentation account for a significant part of production costs. So far most of the work in mutation and selection as well as in the recombinant DNA techniques has been directed to maximizing product levels in the fermenter. I would suggest that genetic engineers should devote at least as much attention to using recombinant DNA technology to make product recovery and isolation more cost-effective. The substantial work done on understanding and using organisms that synthesize extracellular proteins (e.g. *B. subtilis*), exemplified by Professor Young, and especially 'engineering' of extracellular production, illustrates process engineering possibilities. Clearly, there are many other ways in which downstream costs can be reduced by using recombinant DNA techniques.

F. E. Young. I concur that genetic engineers should focus not only on product recovery and isolation but also on ways in which the compounds can be readily immobilized and concentrated. Additionally, we must have close interaction with chemical engineers.

D. E. Brown (*Department of Chemical Engineering, U.M.I.S.T., Manchester, U.K.*). It was reported in the paper that new *B. subtilis* mutants were capable of producing 100 times more enzyme activity than the parent strain. Does this mean that the mutant is producing 100 times the amount of α-amylase protein or producing components that turn existing protein into active enzyme material?

F. E. Young. The mutant is producing one hundred times the amount of α-amylase protein.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES