Impact of Cloning in *Bacillus subtilis* on Fundamental and Industrial Microbiology

The Eighth Griffith Memorial Lecture

By FRANK E. YOUNG

*Department of Microbiology, University of Rochester, Medical Center,
Rochester, New York 14642, U.S.A.*

*(Delivered at the General Meeting of the Society for
General Microbiology on 24 March 1980)*

The Griffith Memorial Lecture is a time for both reflection on and analysis of our branch of science. It marks the commemoration of a notable discovery by a remarkable, thorough, humble bacteriologist (Griffith, 1928). In the 'normal pursuit of science', discoveries are made within the framework of existing paradigms as suggested by Kuhn (1970). Few of us provide the well-documented anomalous observations that eventually precipitate the clash of opposing paradigms. The studies of Griffith, Avery, Watson and Crick stand in stark relief to the landscape of 'Normal Science' as portrayed by Kuhn. Their work led to the ultimate demise of the tetranucleotide hypothesis and the birth of the chemical basis of heredity as encoded by the double helix. In an earlier Griffith Lecture, Pollock (1970) added the discovery of DNA to the 'three really outstanding events in the evolution of human knowledge' as allegedly described by Freud: the Copernican Theory of the Solar System, the Darwinian Theory of Evolution, and the Recognition of Unconscious Mental Processes. I submit that the advent of recombinant DNA technology will introduce a new paradigm that embodies the philosophy, theology, technology and ethics of the manipulation of life. Few advances have so significantly impacted on our society. Accordingly, I shall use the recent work within the *Bacillus subtilis* model system to describe the new advances and potential lines of research that have emanated from this seminal discovery.

Traditional studies with DNA-mediated transformation

Bacillus subtilis was the first non-pathogenic organism to be transformed by DNA (Spizizen, 1958). As with Griffith's tangential migration into the field of genetics from his studies on the epidemiology of infectious disease, Spizizen began his work on transformation from the perspective of another branch of science, viral replication. Following his initial studies on the mechanism of competence (Anagnostopoulos & Spizizen, 1961; Young & Spizizen, 1961, 1963), Spizizen focused on the use of the newly discovered genetic system to unravel the biochemical genetics of sporulation (Spizizen, 1965). The research of others led to the development of many fields of study using the primary tools and techniques of transformation and/or transfection (for a review, see Young *et al.*, 1977). All of these investigations on DNA-mediated transformation, however, were limited to the use of random fragments of DNA and genetic exchange among closely related species. Therefore, the basic steps in recombination were studied with a heterogeneous population of molecules. Early in the development of this model system, transformation was accomplished among closely related bacilli (Marmur *et al.*, 1963). These observations provided an opportunity to study the factors that influenced integration of foreign DNA. The need for extensive nucleotide homology was documented in our laboratory and illustrated in the studies described in Table 1. In these experiments homologous transformation was normalized to

Fig. 1. Fate of heterologous transforming DNA. Double-stranded DNA is represented by two thick horizontal bars. Regions of identical base sequences are solid black. Fine vertical lines indicate proper base pairing between the two strands of DNA. The area enclosed by the parentheses is the site of recognition of a site-specific endonuclease. (From Wilson & Young, 1972*a*)

Table 1. *Interspecific transformation of B. subtilis* 168

DNA from *B. amyloliquefaciens* and *B. amyloliquefaciens* lysogenic for bacteriophage $\phi 105$ was used to transform *B. subtilis* 168 (heterologous transformation). DNA from these transformants was used to transform the same *B. subtilis* recipient (intergenetic transformation). DNA from *B. subtilis* 168 and from *B. subtilis* 168 lysogenic for bacteriophage $\phi 105$ was used to transform *B. subtilis* (homologous transformation). All values are normalized to the frequency of homologous transformation (= 1.0). (Data from Wilson & Young, 1972*a, b*)

Marker	Source of DNA		
	Homologous	Heterologous	Intergenetic
His	1.0	0.0002	0.70
Rif ^R	1.0	0.12	2.57
$\phi 105$	1.0	0.08	0.45

1.0. Note that the initial transformation with heterologous DNA was extremely inefficient. DNA extracted from the transformants that have integrated the foreign gene (intergenetic DNA), however, was usually only slightly less efficient in subsequent transformations than homologous DNA. One exception existed. The gene encoding rifampin resistance transformed *B. subtilis* at a higher frequency than homologous DNA. These and other studies in our laboratory led to the hypothesis that the efficiency of integration of the foreign gene was primarily related to the nucleotide homology at the site of integration, as shown in Fig. 1. Transformation was not substantially influenced by restriction or modification. Procedures to modify the cell to prevent degradation of foreign genes did not improve the frequency of transformation. Once a gene was introduced, it could be transferred only among closely related bacilli. Therefore, the capacity of the cell to become competent (i.e. bind DNA irreversibly) was the rate-limiting step and transformation would occur if there was sufficient nucleotide homology.

Table 2. Transformation of Thy⁻ *B. subtilis* by thyP3 from pCD1 and bacteriophage ϕ 3T

DNA at various concentrations was incubated with a competent culture of *B. subtilis* 168 carrying thyA and thyB. Thy⁺ transformants were selected.

DNA concn (ng ml ⁻¹)	Transformants per ml with DNA from:		
	pCD1	Mature ϕ 3T	Prophage ϕ 3T
500	5.0×10^5	1.0×10^5	ND
50	1.4×10^5	2.9×10^5	10.1×10^3
5	7.8×10^3	3.6×10^4	8.8×10^2
0.5	1.5×10^3	3.9×10^3	3.0×10^1
0.05	2.7×10^2	3.2×10^2	$< 10^1$
0.005	5.5×10^1	3.5×10^1	$< 10^1$

ND, Not determined.

Impact of DNA technology on the understanding of transformation

The discovery of recombinant DNA technology led to a flurry of experimentation in the *Escherichia coli* model system that has revolutionized molecular genetics. Though initially less dramatic, new experiments were performed in the eutransformation system, *B. subtilis*, that enabled investigators to explore the mechanism of recombination. First, using recombinant plasmids, Erhlich (Erhlich *et al.*, 1977) and Young, Duncan & Wilson (Young *et al.*, 1977b) simultaneously and independently established that the gene encoding thymidylate synthetase from bacteriophage ϕ 3T could transform *E. coli* when inserted into plasmids indigenous to *E. coli*. Furthermore, this gene within the chimeric plasmid transformed *B. subtilis* at frequencies approaching the transformation frequency of the gene isolated from *B. subtilis* (Table 2). It was possible to remove the gene encoding thymidylate synthetase from plasmid pCD1 and form the plasmid pCD2 (Fig. 2). This plasmid has a 0.51 megadalton (Md) segment that is homologous to the host chromosome and contains a *Bgl*III site (Duncan *et al.*, 1978). If the gene in the chimeric plasmid showed extensive homology with the chromosome, it was excised from the plasmid and integrated into the chromosome. Under these circumstances, covalently closed circular (CCC) DNA could transform bacteria as well as linear DNA. Thus, it was possible to study the integration of only one gene under carefully defined conditions.

To explore the nature of the integration event, we constructed a series of chimeric plasmids using the gene from bacteriophage β 22 encoding thymidylate synthetase (Fig. 3). All plasmids were constructed from pCD2 which contains the *E. coli* plasmid pMB9 (Duncan *et al.*, 1978). In some of these we used the combination of two site-specific endonucleases that were isolated in our laboratory, *Bam*HI (Wilson & Young, 1975) and *Bgl*III (Wilson & Young, 1977). In the presence of both site-specific endonucleases and ligase, it is possible to select 100% recombinants and exclude the parental molecules, as shown in Fig. 4 (Young & Wilson, 1978). These plasmids were then used to study the integration and expression of foreign DNA by varying the extent of homology to the chromosome of *B. subtilis*. Table 3 summarizes the capacity of the various plasmids to transform and integrate within the chromosome of *B. subtilis*. The frequency of transformation was markedly influenced by the extent of homology. If there was no homology, there was no transformation. Furthermore, transformation occurred only with CCC DNA. Through the use of radioactive probes made by nick translation of pMB9 and pCD4, it was possible to establish that the entire plasmid was integrated into the chromosome of *B. subtilis* by a Campbell-like model resulting in the integration of an extensive stretch of foreign DNA. Furthermore, the reversion frequency of the trait encoded by the foreign gene was proportional to the size of the region of homology. These experiments established the existence of at least two types of transformation events: integration of linear DNA as a func-

Fig. 2. Physical maps of plasmids pCD1 and pCD2. The pMB9 segment of pCD1 and pCD2 is the 3.4 Md fragment between the two *Eco*RI sites. Plasmid pCD2 was formed by excising the fragment between the *Bgl*II sites in pCD1 leaving a small region of homology to the *B. subtilis* chromosome (0.51 Md) that contains a *Bgl*II site. Other genes are cloned with the *Bgl*II site resulting in flanking regions of homology (Fig. 3). (Data from Young & Wilson, 1978)

Fig. 3. Physical maps of plasmids pCD4, pCD5 and pCD6. The gene encoding thymidylate synthetase from bacteriophage β 22 was inserted either directly into the *Bgl*II site of pCD2 or by *Bam*/*Bgl* fusion into pCD2. The location of cleavage by the site-specific endonucleases and the fragment sizes (Md) are included. The fine lines of the circles denote the DNA segment(s) from bacteriophage ϕ 3T that are homologous to the chromosome of *B. subtilis*, the moderately thick lines denote DNA from pMB9, and the thickest lines denote DNA from bacteriophage β 22.

tion of nucleotide homology and integration of either the entire plasmid CCC DNA or merely the homologous segment depending on the extent of nucleotide homology. These models are summarized in Fig. 5. Recent studies (Mottes *et al.*, 1979) indicate that the plasmid DNA involved in transformation is a concatamer. Possibly, an intracellular recombinational event involving more than one copy may be required. Alternatively, extensive degradation during uptake may reduce the efficiency of monomers (Contente & Dubnau, 1979; Mottes *et al.*, 1979). The exact mechanism of this integration remains to be elucidated.

Fig. 4. *Bam/Bgl* fusion. Incubation of DNA with site-specific endonucleases *Bam*HI and *Bgl*II in the presence of ligase produces a unique site and results in survival of only the recombinant molecules.

Table 3. Transformation of *B. subtilis* 168 by chimeric plasmid DNA

A competent culture of *B. subtilis* 168 carrying *thyA* and *thyB* was incubated with various preparations of chimeric plasmid DNA carrying a gene encoding thymidylate synthetase. The gene encoding thymidylate synthetase was derived from bacteriophage ϕ 3T in pCD1 and bacteriophage β 22 in pCD4, pCD5 and pCD6. Note pCD5 does not have a homology with the chromosome of *B. subtilis* and lacks the 0.51 Md homologous segment present in pCD4 and pCD6. Relative transformation frequencies are normalized to the transformation of the recipient with *B. subtilis* chromosomal DNA (= 1.0). (Data from Duncan *et al.*, 1978)

Plasmid	Homologous segment		Relative transformation frequency	
	Region	Origin	CCC	Linear
pCD1	<i>thyA</i>	ϕ 3T	0.83	0.75
pCD5	None	None	0	0
pCD4	0.51 Md	ϕ 3T	0.0008	0
pCD6	0.51 Md	ϕ 3T	0.0014	0

To obtain a foreign gene analogous to the *B. subtilis* gene encoding thymidylate synthetase, we chose a completely heterologous system. We concluded that the best control for transformation studies and physical analysis was a gene isolated from *E. coli* that was cloned in an *E. coli* plasmid. Because there was only 4% DNA:DNA hybridization between *E. coli* K12 and *B. subtilis* (Lovett & Young, 1969), we anticipated that the *E. coli* gene encoding thymidylate synthetase could not transform *B. subtilis*. Accordingly, chromosomal DNA was isolated from *E. coli*, introduced into pMB9 and used to transform *Thy*⁻ auxotrophs of *E. coli* to *Thy*⁺. The chimeric plasmid pER2 contains only *E. coli* DNA with no detectable homology to the *B. subtilis* chromosome by the Southern technique (Rubin *et al.*, 1980). To our surprise, the frequency of transformation was approximately one-third the frequency of transformation with *B. subtilis* DNA. Transformation was markedly reduced if the DNA was made linear with site-specific endonucleases (Table 4). Three experiments were performed to determine whether the DNA existed as a plasmid or was integrated into the chromosome of the cell. First, the DNA from the transformants was analysed on sucrose gradients. As shown in Fig. 6, the *Thy*⁺ transforming activity is located within the peak of chromosomal DNA and not in the portion of the gradient where plasmid DNA sediments. Second, a radioactive probe made from the donor plasmid hybridized to endonuclease digest of the chromosome of the transformants at two distinct bands in the chromo-

Fig. 5. Plasmid-mediated transformation. A: Transformation of a recipient by a plasmid; the plasmid is maintained if there is no homology or if the recipient carries *recE*. B: Transformation of a recipient by a plasmid with extensive homology to the chromosome, such as pCD1. C: Transformation of a recipient by a plasmid with limited homology; the homologous segment is denoted by ■.

Table 4. *Thy*⁺ transforming activity of chimeric plasmids in *B. subtilis* 168

A competent culture of *B. subtilis* 168 carrying *thyA* and *thyB* was incubated with various preparations of plasmid and chromosomal DNA (1 µg ml⁻¹). *Thy*⁺ transformants were selected. One sample of DNA was treated with *Bam*HI site-specific endonuclease prior to transformation. (Data from Rubin *et al.*, 1980)

Donor DNA	Transformants per 10 ⁸ cells	
	No enzyme treatment	<i>Bam</i> HI treatment
<i>B. subtilis</i> 168 chromosomal	3.0 × 10 ⁵	ND
<i>E. coli</i> K12 chromosomal	< 10	ND
pER2 (pBR322+ <i>E. coli</i> <i>ThyA</i>)	6.0 × 10 ⁴	4.5 × 10 ²
pER4 (pBR322- <i>E. coli</i> <i>ThyA</i>)	< 10	ND
pER5 (pMB9+ <i>E. coli</i> <i>ThyA</i>)	4.1 × 10 ⁴	6.0 × 10 ²

ND, Not determined.

Fig. 6

Fig. 7

Fig. 6. Transformation of *B. subtilis* with DNA from pER2-transformed cells. (a) 40 μ g DNA isolated from *B. subtilis* RUB890 (Trp⁺ *thyA thyB*) that had been transformed to Thy⁺ with pER2 DNA was layered on 9 ml CsCl/ethidium bromide and adjusted to a refractive index of 1.398. The mixture was centrifuged on a Beckman model L3-50 ultracentrifuge with a Ti50 rotor at 40000 rev. min⁻¹ at 15 °C for 60 h. Six drop fractions were collected from the top of the gradient and 50 μ l of each sample was assayed for its ability to transform *B. subtilis* RUB830 to either Thy or Trp independence. (b) The experiment was similar to that in (a) except that the source of DNA was 0.92 μ g plasmid pER2 plus RUB890 (Trp⁺ *thyA thyB*) DNA. $\circ$, Trp⁺ transformants; $\square$, Thy⁺ transformants. (Data from Rubin *et al.*, 1980)

Fig. 7. Hybridization of pER2 with DNA from Thy⁺ transformants of *B. subtilis*: (A) *Eco*RI digest of pER2; (B) DNA from *B. subtilis* RUB830 transformed with pER2 DNA; (C) *Eco*RI digest of pER2-transformed *B. subtilis* DNA; (D) Autoradiograph of radiolabelled pER2 hybridized to pER2-transformed *B. subtilis* DNA; (E) Autoradiograph of radiolabelled pER2 hybridized to *Eco*RI digest of pER2-transformed *B. subtilis* DNA. (From Rubin *et al.*, 1980)

some (Fig. 7). In other experiments, it was possible to demonstrate that the pMB9 segment as well as the *thyA* gene were incorporated into the chromosome. Therefore, a plasmid without substantial homology to the chromosome of *B. subtilis* transformed the recipient cells at a high frequency. These and other studies in our laboratory indicate that additional factors besides extensive nucleotide homology must influence recombination. The integration of the plasmid vector as well as the foreign gene is not restricted to the *B. subtilis* chromosome. Yeast can be transformed by a chimeric plasmid that consists of ColE1 and the *leu* gene from yeast. In one class of recombinants, the chimeric plasmid was integrated into the yeast chromosome (Hinnen *et al.*, 1978).

The basis for this unusually high frequency of recombination of *E. coli* genetic material into the *B. subtilis* chromosome is not understood. It must be recalled that the insertion into the chromosome occurs at a single, or at most, two sites (Rubin *et al.*, 1980). Additionally, all recombinants showed the same pattern of integration into the recipient chromosome as evidenced by Southern hybridization. Finally, there was no evidence of homology between the chromosome of *B. subtilis* and this vector, at least as measured by the relatively crude Southern hybridization methodology. Studies on the extent of hybridization of DNAs from various bacilli with *B. subtilis* included, as a control, a sample of *E. coli* K12 DNA. As mentioned earlier, a hybridization of 4% was noted between *E. coli* K12 and *B. subtilis* in

Fig. 8. Bacteriophage SPO2 endonuclease fragment map. The *BglI*, *BglII*, *EcoRI*, *SalI*, *SmaI* and *XbaI* sites were ordered using the heat-dissociable junction as an origin. Fragment sizes are in Md. (Data from Graham *et al.*, 1979)

the DNA:DNA filter binding technique (Lovett & Young, 1969). Although this degree of binding was originally considered to be a noise level, its significance may need to be reassessed. Assuming that the double-strand form was required for transformation, it is most plausible that a site-specific recombination had occurred. Furthermore, it is postulated that the number of sites for recombination are limited in the case of pCD4, pCD6, and pER2. Because *E. coli* plasmids used to construct the vectors are similar, the site of recombination must be in the foreign gene or the ϕ 3T segment that is homologous to chromosomal DNA. The reason for the high frequency of transformation of the heterologous gene encoding thymidylate synthetase in pER2 is unclear. In addition to the double site-specific type of recombination (Low & Porter, 1978), there may be a protein associated with the *E. coli* gene encoding thymidylate synthetase that markedly stimulates recombination. Studies are in progress to elucidate the mechanism of this unexpected event. Because *B. subtilis* can incorporate a variety of plasmids, as initially discovered by Ehrlich (1977) and documented by Dubnau and co-workers (Gryczan & Dubnau, 1978), it is likely that selective pressure could result in substantial recombination of chimeric plasmids with the chromosome of *B. subtilis*. Thus, foreign genes could be scavenged by *B. subtilis*.

In addition to a new emphasis on plasmid biology in *B. subtilis*, the advent of cloning technology has stimulated the investigation of bacteriophages as potential cloning vectors. Three major candidates have emerged: bacteriophages ϕ 3T, SPO2 and ϕ 105 (Young & Wilson, 1978). Bacteriophage ϕ 3T was originally suggested as a potential candidate for cloning experiments because of the presence of a gene encoding thymidylate synthetase (*thyP3*) within its genome (Young, 1976). The *thyP3* gene is similar to the chromosomal *thyA* gene as evidenced by genetic and biochemical studies (M. T. Williams & F. E. Young, unpublished observations); therefore, it can be readily used to select lysogens containing foreign DNA. The availability of well-characterized deletion mutants and the establishment

Table 5. Comparison of the genomes of bacteriophages SPO2 and SPO2·Clh2

Preparations of DNA from bacteriophages SPO2 and SPO2·Clh2 were digested with the site-specific endonucleases *Bgl*II and *Eco*RI, electrophoresed and the sizes of the fragments were determined.

SPO2+ <i>Bgl</i> II		Clh2+ <i>Bgl</i> II		SPO2+ <i>Eco</i> RI		Clh2+ <i>Eco</i> RI	
Fragment	Md	Fragment	Md	Fragment	Md	Fragment	Md
A	(8·31)	A	(7·80)	A	(16·04)	A	(4·75)
B	4·11	B	4·99	B	4·74	B	3·39
C	3·12	C	3·12	C ₁ C ₂	1·62	C	2·84
D	2·29	D	2·16	C ₁	0·78	D	2·28
E	2·11	E	2·07	C ₂	0·75	E	2·14
F	1·62	F	1·66	D	0·66	F	1·94
G	0·58	G	0·54			G	1·26
H	0·46	H	—		23·06	H	0·80
I	0·45	I	—			I	0·67
						J	0·63
						K	0·52
	23·05		22·34				21·22

of a physical map of this virus (Cregg & Ito, 1979) should greatly aid the analysis of cloned segments of foreign genes.

The genome of bacteriophage SPO2 was also characterized by physical techniques (Yoneda *et al.*, 1979*b*). Although deletion mutants were obtained, all of them were confined to the region of the *Bgl*II site (Fig. 8). In other studies it was possible to extend the host range of this virus by plating 10¹⁰ phage propagated on *B. subtilis* 168 on *B. amyloliquefaciens*. One of these phages appeared to be a natural chimeric between bacteriophage SPO2 and an as yet unidentified resident bacteriophage from *B. amyloliquefaciens*. The differences in the site-specific endonuclease pattern of the parent SPO2 and the recombinant SPO2·Clh2 are shown in Table 5. Despite these changes *sus* mutants of SPO2 can be rescued by DNA from SPO2·Clh2 in superinfection marker rescue experiments. These are only a few examples of the fundamental studies that have been stimulated by the attempt of investigators to develop cloning vectors.

Impact of cloning on industrial microbiology

The work which followed Griffith's discovery (1928) was limited by the heterogeneity of the DNA preparations. While this hindered fundamental molecular genetic studies, it was a more significant impediment to strain improvement for industrial purposes. The advent of cloning in *B. subtilis* has enabled us to isolate pure genes and to devise a variety of non-selective methods to identify the desired clone. Because of the major commercial importance of bacillus products, these organisms have been used for the production of a variety of exoenzymes (Priest, 1977) and could be tailored for excretion of eukaryotic products. Furthermore, these organisms do not produce disease in the uncompromised host (Young *et al.*, 1977*b*) and are consumed in large quantities in the Orient (Y. Yoneda, personal communication). Therefore, *B. subtilis* should provide an ideal organism for production of a variety of prokaryotic and eukaryotic proteins. Currently, three major strategies are under investigation (Table 6).

First, plasmids from *Staphylococcus aureus* introduced into *B. subtilis* have been used to clone foreign genes (Keggins *et al.*, 1978; Gryczan & Dubnau, 1978). If the *recE* mutation is present within the recipient cell, homologous genes can also be cloned. In the absence of such a mutation the homologous gene is integrated into the chromosome of the host. The

Fig. 9. Construction of regions of homology for scaffolding. The vector integrated in Fig. 5 C provides a region of homology for another chimeric plasmid through a common portion within the two vectors. Integration of one vector occurs within the other resulting in the introduction of a new genetic trait.

Table 6. Major cloning strategies

Method	Major advantages	Major limitations
<i>S. aureus</i> plasmids containing homologous or homologous DNA	Multiple copies Selective markers	Competent cells required <i>RecE</i> mutation necessary
<i>E. coli</i> plasmids with homologous segment and heterologous DNA	Integration with host chromosome Scaffolding potential	Competent cells required Single gene copy
Bacteriophage vectors	Competent cells not required Multiple genes introduced into recipient cells	Host range limitations Induction of lysogens leading to lysis

development of a chimeric plasmid by Ehrlich (1978) that replicates in both *B. subtilis* and *E. coli* has greatly increased the potential of this cloning system.

Second, it is possible to integrate foreign genes into the chromosome of *B. subtilis* through the development of chimeric plasmids with limited regions of homology as described in the previous section. In these experiments it is essential that the ratio of homologous to heterologous DNA be low to preclude excision of the homologous region. The addition of a new foreign segment of DNA should provide an opportunity for the introduction of additional segments of DNA through recombination in the new region of homology, as shown in Fig. 9. For example, the first gene introduced would be the gene for thymidylate synthetase from *E. coli* within the vector pBR322. Subsequently, another gene with a selective trait or a non-selective trait cloned in the same vector could be used to transform the first recipient. Thus, any gene previously cloned in pBR322 in *E. coli* could be readily integrated into the chromosome of *B. subtilis*. Such procedures could permit the industrial microbiologist to use a safer strain for larger scale industrial processes involving eukaryotic gene products. Because *B. subtilis* lacks endotoxin (Young *et al.*, 1977*b*) that is found in the cell wall of Gram-negative microbes, it is a particularly attractive cloning system for human consumption.

Third, it is possible to clone both homologous and heterologous genes in bacteriophage ϕ 3T or the closely related bacteriophage ρ 11. The procedures for cloning heterologous non-selective genes are shown in Table 7. The use of congression to select clones that may have

Fig. 10. Polyacrylamide gel profiles of α -amylase activity. The activity of amylase from *B. amyloliquefaciens* strain H (denoted by H), *B. subtilis* strain M (denoted by M), *B. subtilis* var. *amyloliquefaciens* (denoted by S) and six transformants was determined as described by Yoneda *et al.* (1979a). Recipient, *B. subtilis* strain M; donor DNA, bacteriophage ϕ 3T(Amy⁺); the Amy⁺ gene was cloned from *B. amyloliquefaciens* strain H. Note each transformant produces both the donor and the recipient α -amylase.

Table 7. Cloning of *B. amyloliquefaciens* α -amylase in *B. subtilis*

1. With *Bgl*III, limit digest ϕ 3T DNA and *B. amyloliquefaciens* strain H DNA
2. Heat inactivate *Bgl*III, mix the DNAs, ligate with T4 DNA ligase
3. Transform a MO7A Thr⁻ (Amy⁻ Aro⁻ Thr⁻) ϕ 3T lysogen with MO7A (Thr⁺ Amy⁻) DNA and DNA from step 2
4. Select Thr⁺ using Spizizen's minimal + aro + starch
5. Use I₂-halo to detect the Amy⁺ transformants among the Thr⁺ transformants
6. Check the Amy⁺ transformants: (a) level of amylase production
(b) type of amylase

incorporated the foreign non-selected gene is particularly relevant to industrial microbiology. The analysis of as few as 200 clones should reveal the identity of at least five colonies that also contain the non-selected trait. We have successfully used this methodology to introduce the gene encoding α -amylase from *B. amyloliquefaciens* into *B. subtilis* 168 (Yoneda *et al.*, 1979a). The α -amylase from *B. subtilis* can be readily distinguished from the α -amylase of *B. amyloliquefaciens* on gel electrophoresis, as shown in Fig. 10. Analysis of the enzyme activity of various strains of *B. subtilis* carrying different combinations of genes encoding α -amylase indicates that yields of amylase approaching the original donor fermentation strain can be developed merely by the addition of a single gene. By the introduction of appropriate mutations into the recipient strain yields can be improved (Table 8). It is anticipated that the introduction of multiple gene copies encoding different α -amylases coupled with genes regulating excretion of these exoenzymes should further increase fermentation yields. These general procedures should enable cells to be infected by the appropriate viruses carrying recombinant DNA for a variety of fermentation processes. Thus, one mother strain can be eventually used for multiple fermentations.

Recently we have developed a certified HV1 strain (RUB331) of *B. subtilis* (S. L. Mottice, G. A. Wilson & F. E. Young, unpublished observations). This asporogenic mutant will

Table 8. Production of α -amylase by various strains of bacilli carrying cloned genes

Various strains of *B. subtilis* were lysogenized with bacteriophage ϕ 3T carrying the gene encoding α -amylase from *B. amyloliquefaciens* ϕ 3T(Amy⁺). The activity of α -amylase was measured in each lysogenic and control strain.

Strain	Relevant genotype*	Additional traits*	α -Amylase (units ml ⁻¹)
<i>B. subtilis</i> 168	<i>amyE</i> ⁺ <i>amyR</i> 1	None	9.6
<i>B. subtilis</i> 168	<i>amyE</i> ⁺ <i>amyR</i> 2	None	46.0
<i>B. subtilis</i> 168	<i>amyEO</i> 7 <i>amyR</i> 2	None	0.71
<i>B. subtilis</i> 168	<i>amyEO</i> 7 <i>amyR</i> 2	ϕ 3T(Amy ⁺)	82.1
<i>B. subtilis</i> 168	<i>amyEO</i> 7 <i>amyR</i> 2	<i>papM</i>	0.01
<i>B. subtilis</i> 168	<i>amyEO</i> 7 <i>amyR</i> 2	ϕ 3T(Amy ⁺) <i>papM</i>	352.0
<i>B. subtilis</i> 168	<i>amyE</i> ⁺ <i>amyR</i> 3	<i>amyS papS</i>	237.06
<i>B. subtilis</i> 168	<i>amyE</i> ⁺ <i>amyR</i> 3	<i>amyS papS</i> ϕ 3T(Amy ⁺)	939.2
<i>B. amyloliquefaciens</i>	Amy ⁺	None	625.0

* *amyE* is the structural gene for α -amylase; *amyR* is the specific regulator of α -amylase; *amyS* is the gene regulating the production of α -amylase; *papM* and *papS* are the genes regulating the production of α -amylase and protease.

Fig. 11

Fig. 12

Fig. 11. Viability of *B. subtilis* strains RUB331 and RUB830. Cells were grown in M broth [0.1 mM-Fe(NO₃)₃] with and without the addition of 0.5% (w/v) glucose. 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 .

Fig. 12. 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 4.28×10^6 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 to 20 min) was 1×10^{-5} on day 2, 8×10^{-4} on day 4, 2×10^{-4} on day 6, 5×10^{-5} on day 10, and 1×10^{-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 likely arose from leakiness rather than from reversion of the mutation.

undergo transformation at a frequency of 1 to 2% with both plasmid and chromosomal DNA. Furthermore, it does not withstand desiccation or survive appreciably in broth (Fig. 11). The decreased survival in broth in the presence of glucose is probably related to the activation of autolysins (Brown & Young, 1970). Finally, studies in soil have revealed that this organism is unlikely to survive if it escapes from the laboratory. In these experi-

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Fig. 13

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Fig. 14

ments we have examined the ability of the organism to survive in flower pots, as shown in Fig. 12. Therefore we have developed a suitable HV1 system that will permit cloning of eukaryotic genes in *B. subtilis*. Our initial study demonstrating the cloning of the yeast gene encoding cytochrome *c* will be described later in this meeting.

The *B. subtilis* model system is now at the threshold of major utilization in industrial microbiology. Whereas the basic well-characterized *E. coli* model system could be readily adapted to the development of recombinant molecule technology, the rudimentary knowledge of molecular genetics in *B. subtilis* hindered its utilization. Therefore, vehicles and vectors had to be developed to permit the orchestration of novel genes within the harmonic development of cellular functions. Three unique aspects of the *B. subtilis* system should make it particularly useful in cloning. First, the organism is non-pathogenic and proteins produced in *B. subtilis* can be readily purified without fear of contamination by endotoxin. Second, the organism excretes large amounts of extracellular products. Studies are in progress to perfect the extracellular excretion of novel proteins. The combination of appropriate genes that influence excretion of products should yield high-producing mother strains. Third, a single mother strain could be used for many different fermentations. The development of viral cloning systems allows the use of mutations in the host cell that stimulate high productivity of extracellular proteins such as protease and amylase but are accompanied by a concomitant reduction in transformability and autolysis (Yoneda *et al.*, 1973; Yoneda, 1980). Therefore, viral systems will continue to be developed to augment the scaffolding approach to genetic engineering.

The advent of recombinant DNA technology has ushered in a plethora of regulations of basic and applied genetics. In large measure, these regulations have resulted from the fears of the scientific and lay public as we begin to grapple with the advantages and risks of a new technology. Cartoons in the American press have captured some of the fantasy about cloning human beings. Two representative cartoons lampooning this concern are shown in Figs 13 and 14. It is important to remember that society's deepest anxieties can often be captured through the pen of the cartoonist.

The government of the United States has agonized, as has that of Great Britain, over the balance of regulation of research and freedom of inquiry. As a member of the US Recombinant DNA Advisory Committee, I am convinced of the wisdom of public participation in decision-making, but am appalled at the insufficiency of our current knowledge. I submit that this emerging paradigm will embody government, law, medicine, science,

theology and society in a fashion unparalleled in other scientific revolutions. As we end the twentieth century, mankind will possess the tools for gene therapy and grasp the potential for manipulation of his own genome. Accordingly, it is imperative that we do not rely on science alone, but synthesize our technology with ethics and theology. As we enter the twenty-first century, we must come to grips with the questions of the meaning of life, the purpose of life and the dignity of life. The production of human and mammalian products in bacteria has been accomplished in the laboratory, and scale-up industrial applications are underway. The synthesis of products normally found in one animal by other animal cells is routine, as exemplified by the synthesis of ovalbumin by mouse cells (Lai *et al.*, 1980). Gene therapy will soon be a reality. In the preface of the first book on the manipulation of the genome of mankind in 1965, entitled *The Control of Human Heredity and Evolution*, Tracy Sonneborn commented:

'The human problems raised by these new possibilities are not fundamentally different from the problems Huxley put forcibly before the public. They are problems of morals, ethics, religion, and politics. They are problems of how knowledge, and the power that knowledge confers, will be used. They could be used for good or for ill, for the enslavement or the liberation of man. How they will be used obviously will not be decided by scientists alone. Nor should this be decided alone by professional politicians or by theologians or by philosophers or by moralists. It should be decided by an enlightened and broadly based public opinion.'

Fifteen years have elapsed since this far-sighted comment. There is little time remaining before these grave ethical issues impact on our society. I hope this summary of the development of the *B. subtilis* model system will stimulate us to analyse these questions.

It is with great pleasure that I acknowledge the contributions of Gary A. Wilson, Scott Graham, Edward Rubin, Craig Duncan, Marshall Williams and Yuko Yoneda. Their efforts made this presentation possible and their friendship has heightened the quality of science in our laboratory. The more recent research discussed in this paper was supported by a grant from Miles Laboratories.

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