ADVANCES IN THE GENETICS OF INDUSTRIAL MICROORGANISMS

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MICROBES have been used for several decades to synthesize molecules of great complexity like antibiotics and other secondary metabolites[1]. In the next phase, biotechnology will have its greatest impact in converting low grade biomass, such as cellulose, into products of higher value. In the past decade new opportunities for biotechnologists have emerged at an unprecedented pace[2]. Since various microbes are being used as factories for synthesizing commercial products, knowledge of their biology aids in their precise manipulation to obtain desired results[3, 4].

Monoclonal antibodies are progressively being used as diagnostic reagents. Nucleic acid hybridization probes for detecting diseases etc will soon make an impact in human diagnostics[5]. Aspartic acid and phenylalanine are being produced in enzyme-based reactors for aspartame, a dipeptide ester 100 times sweeter than sucrose. Chymosin (rennin), a milk-clotting enzyme of the calf stomach has been produced in various microorganisms[6] and will soon be commercialized. Enzymes such as lactases, proteases, amylases, lipases, pectinases and amyloglucosidases will be produced cheaply by microorganisms constructed by recombinant DNA methods[6]. The ability to produce enzymes in large quantities at low cost and the technology to modify the existing enzymes and tailor them to the requirements of specific industrial processes will make their use practical.

The ability to write in the language of DNA and the arrival of the gene machine have made it possible to synthesize oligonucleotides of significant lengths. This allows targeted mutagenesis to obtain point mutations which in turn facilitate directed modification of the structure of protein molecules with enhanced functional capabilities[7]. Synthetic oligonucleotide directed mutagenesis of the coding sequence for proteins has already been used to accomplish replacement of threonine with alanine in tyrosine-t RNA synthetase which reduces Km by a factor of 2. Cetus scientists have already replaced cysteine-17 with serine in human Beta interferon to obtain improved stability and yield. Lysozyme T4 has been made thermostable by this methodology and Genecor scientist David Estell has modified subtilisins to obtain variants with altered substrate specificity, pH optimum and an increased total efficiency. Catalytic activity of glucose isomerase has been improved by Amgen Scientist B. Ratzkin.

A – *ESCHERICHIA COLI*

Genetic engineering techniques have converted *E. coli* into an industrial microorganism for the production of a variety of pharmacologically active proteins. Many vectors that efficiently express any gene coding sequence in *E. coli* have been constructed. Increased expression has been obtained *via* the use of vectors which provide tandem promoters, downstream terminators and varied ribosome-binding sites. The effects of translational coupling on gene expression, secondary structure in RNA, codon utilization and position with regard to translational regulation appear important.

E. coli rapidly degrades abnormal proteins which may arise by mutations, gene fusions, or translational errors. Many proteins cloned in bacteria are rapidly hydrolyzed by 6 soluble proteases named proteases Do, Re, Mi, Fa, So and La. Besides, two other membrane-associated proteases; *E. coli* contain two soluble endoproteases named Ci and Pi that degrade smaller polypeptides like insulin.

Protease La which is a heat-shock protein catalyzes the initial cleavages of proteins and ATP

in a coupled process. Protein substrates stimulate ATP hydrolysis. Two ATPs are utilized per peptide bond cleaved. The overall rate of proteolysis in *E. coli* is controlled by the *htpR* locus. Protease La content rises under various conditions that cause the production of large amounts of abnormal proteins within cells (*e.g.* exposure to high temperatures, amino acid analogs, or certain plasmids). Induction of this protease thus may help protect the host against accumulation of abnormal polypeptides.

Human insulin is commercially prepared by combining the A and B chains purified from separate cultures of *Escherichia coli* containing recombinant plasmids by Eli Lilly and Company, Indianapolis USA[2]. The insulin peptides are synthesized from Ptrp as a part of fusion proteins, containing portions of the tryptophan operon leader peptide and E protein joined to insulin chain by methionine residue. The activity of the trp R gene product regulated the synthesis of heterologous proteins from the tryptophan operon controlling region. This was achieved by using a host which is temperature-sensitive in trp R gene product. Cells can be propagated to very high density in the tryptophan-rich medium at low temperature whereupon the synthesis of heterologous protein could be derepressed by raising the temperature to 42°C. This allowed the growth of cells to a high density before the onset of synthesis of excessive protein which may be toxic to the producing host.

Chemically synthesized DNA sequences encoding insulin-like growth factors (IGFs, somatomedins) I and II were fused to parts of the *trp E* structural gene to create fusion proteins of 12 KD, 18 KD and 29 KD in size. The highest level of expression was obtained with the thermoinducible runaway-replication plasmid when coding sequence for 12 KD fusion protein was cloned behind the trp promoter and ribosome binding site. The 12 KD protein accumulates as denatured aggregated granule, representing about 30 percent of the total cell protein. (J. K. Epp and J. A. Hoskins, Eli Lilly and Co.)

Methionyl bovine growth hormone contains N-terminal sequences that do not allow for efficient translation of its m-RNA[8]. B. E. Schoner et al (Eli Lilly Co.) solved this problem by introducing additional codons 3′ to the initiating AUG codon. A two-cistron system consisting of the codons which enhance the expression of the bovine growth hormone into the first cistron and the coding sequence for methionyl bovine growth hormone into the second cistron, produced expression level of up to 30% of total cell protein.

One of the major difficulties with the production of vaccines by the genetically engineered microorganisms has been the recreation of viral antigenic sites from the largely denatured proteins produced. They were overcome with the foot-and-mouth disease VPI, the hepatitis B surface antigen and the herpes type I and II glycoprotein D. High expression in mammalian-cell culture, using SV40 early promoter vector and amplification using DHFR gene may be used to produce antigenic proteins, in which most antigenic sites are properly exposed[2].

While certain proteins produced in *E. coli* are toxic, others have a relatively low toxicity but are insoluble. Large amounts of insulin, calf rennin, growth hormone and other desirable protein accumulated in protein bodies in a denatured form[9]. Inclusion bodies made in *E. coli* as a result of high level of expression of calf prochymosin, are composed of multimers of prochymosin molecules interlinked to a large extent by disulfide bonds. Reduced prochymosin monomers are also bound to the surface of the body in contact with the cytoplasm. Due to the intermolecular disulfides prochymosin is very hard to solubilize from *E. coli* (Shoemaker, J. M. and Marston, F. A. O., Celltech Ltd., Slough, U.K.). These proteins have to be purified, unfolded and refolded into biologically active three-dimensional architecture before use. Microbial host for producing recombinant proteins is very important. Different pathways of protein processing and the presence of different proteases in various organisms may produce the protein of interest in different states. Therefore, commercially, each protein may have to be produced in a suitable microbial host.

B–*BACILLUS SUBTILIS*

Bacillus is interesting both academically and industrially. It forms spores, a model of cell

differentiation. It also makes a large number of commercial products, insecticides, antibiotics and enzymes. *B. thuringiensis* makes insecticidal proteins[2, 10].

The fusion of *E. coli* β-galactosidase structural gene lac Z, with promotor and controlling elements of any other gene from any source, provides a convenient and well exploited enzymatic assay for dissecting the nature of genetic elements controlling gene expression. Andreoli, P. M. and coworkers (Gist-Brocades N. V. Delft) constructed a plasmid pGBG3415 which can be used to isolate and identify restriction fragments with promoter activity in *B. subtilis*. *B. licheniformis* DNA fragments with regulatory region were inserted in proper orientation in pGBG 3415 in the ECORI, Sma I, or BamHi restriction site of the first 24 base pairs of the lacZ structural gene to achieve its expression.

Spo VG gene was fused to lacZ gene and then introduced into Spβ phage. This gene construction was put in Bacillus chromosome by prophage so that the activity of the promoter can be monitored by assaying β-galactosidase[11]. M. A. Sullivan (Southern Research Institute, Birmingham, Alabama) has constructed versatile vectors which allow efficient isolation and characterization of *lacZ* gene fusions in *B. subtilis*. These vectors replicate and confer ampicillin resistance in *E. coli* and have coding sequence of *lacZ*. A DNA fragment containing the regulatory region and eight aminoacids of the leader sequence of the extracellular serine protease of *B. subtilis* (*sprE*) has been inserted at the restriction sites in the N-terminal of LacZ gene, in frame with the remainder of LacZ coding sequences. Transformants arise by integration *via* homology with the cloned fragment, resulting in only one copy of fusion per cell. In the integrated form, β-galactosidase was synthesized in the idiophase in sporulation medium. Elevated amounts of β-galactosidase is produced during logarithmic growth when fusion is present on a multicopy plasmid but still 3- to 4-fold induction occurs when cells enter stationary phase.

The nuclease gene (nuc) of *Staphylococcus aureus* has been expressed in *B. subtilis* either from its own promoter and translation signals or from a combination of a *Bacillus* promoter, ribosome binding site and signal peptide sequence[12]. Levels of nuclease secreted were about 50 mg/liter depending on the culture medium, strain and growth conditions. These studies with secretion of *S. aureus* nuclease in *B. subtilis* will provide the knowledge which could be used for producing heterologous proteins in *B. subtilis*.

Another promoter-cloning plasmid for *B. subtilis* consists of a promoterless gene, *cat*-86, inserted in pUB110 at a site that prevents vector-initiated transcription[13]. A 21 bp multicloning site linker located 144 bp upstream from *cat*-86 in pPl703 permits the cloning of promoters into unique *EcoRI*, *BamHI*, *SalI* and *PstI* sites. In the promoter-containing derivatives of pPL703 *cat*-86 expression is inducible by chloroamphenicol. Inducibility is independent of the promoter which is chosen to activate the gene, and inducibility is retained when *cat*-86 is replaced with the *lacZ* gene. Sequences essential to inducibility therefore reside in a 144 bp segment located between the site of promoter insertion and the *cat* gene. Chloroamphenicol inducibility is due to post-transcriptional regulation. Chloramphenicol-modified ribosome interact with *cat* mRNA to block formation of secondary structure that would, in the absence of the drug, sequester the *cat* ribosome-binding site[13].

Bacilli secrete proteins in the medium from where it can be easily purified. Protein export in bacteria shares features ascribed to protein translocation in the endoplasmic reticulum and mitochondria in eucaryotic cells[14]. There is more than one mechanism for protein export. Secretion pathways have been conserved. Bacterial signal sequence can transport eucaryotic proteins and *vice versa*. Small peptide called "signal peptide" decides whether protein is going to be secreted. However, the knowledge of the precise mechanism is limited even though more than seventy mutations in signal sequence have been characterized in *E. coli* and 25 gene products have been defined. It is not yet possible to formulate the rules of protein export.

In bacteria, a unique modification and processing system for the biosynthesis of the membrane-bound lipoproteins with covalently linked fatty acid and glyceride exists. A major

consensus sequence (leu-ala-gly-cys) at the N-terminal end of these prolipoproteins constitutes the recognition site for glyceryl transferase, the first modifying enzyme. The signal peptide is cleaved from the modified lipoprotein by a unique signal peptidase. The signal sequence of *bacilli* differs from typical sequences in gram-negative organisms as the N-terminal segment is more positively charged and frequently longer. The hydrophilic stretch of small and polar residues continues beyond the probable signal peptide with further processing to form the mature extracellular protein. For the few identified products of signal peptidase action, the sequences involved are consistent with the requirements for cleavage in gram-negatives. Both a general and a lipoprotein-specific enzyme are present. Processing by proteases is both an essential step in maturation and a major impediment in the production of cloned foreign proteins. *B. subtilis* membranes contain four peptidases, one neutral and one alkaline protease. Leader peptidase gene has been cloned and the enzyme purified and isolated.

STREPTOMYCES

Streptomycetes produce most of the commercially important antibiotics, enzymes and biotransformation products and grow well on a variety of inexpensive substrates[15, 16]. Their genetic manipulation may allow isolation of genes involved in antibiotic synthesis and their introduction into other microbes[17–19].

Structural gene for phenoxazinone synthase (PHS), a key enzyme in the biosynthesis of actinomycin in *S. antibioticus*, has been cloned using the plasmid vector, pIJ 702[20]. High levels of this enzyme were detected after transformation of *S. lividans* with the resulting recombinant plasmid.

Two other cloned DNA fragments from *S. antibioticus* which neither overlap the 2.4 kb structural gene nor code for PHS related proteins induce production of active PHS after transformation of *S. lividans* with the corresponding recombinant plasmids[21]; these cloned fragments may activate a normally "silent" PHS gene in *S. lividans* which has been identified by Southern blotting using the cloned *S. antibioticus* PHS gene as a probe.

Strains of *S. antibioticus* transformed with the high copy-number plasmid, PIJ 702, which carried tyrosinase gene grow less vigorously but exhibit 3-fold increase in the levels of secreted tyrosinase and 150-fold increase in intracellular tyrosinase in comparison with parental strains lacking tyrosinase on a plasmid. However while parent *S. antibioticus* exports more than 95% of tyrosinase, less than 20% is exported in strains harboring PIJ 702[22].

Over 21 kb of genomic DNA was cloned which "complemented" several specific mutations in the *red* gene cluster which codes for the production of the red pigmented antibiotic undecylprodigiosin by *S. coelicolor* A3(2). Mutational cloning in the actinophage Ø31 suggests two transcription units in the *red* region (Feitelson, J. S., American Cyanimide Co., Pearl River, NY).

A 24.5 kilobase vector; PIJ 922; with thiostrepton resistance as a selectable marker was used to clone in *S. coelicolor* actinorhodin genes which were on a 34 kb insert; this fragment harbors genes for resistance to and biosynthesis of actinorhodin. When the genes are transferred into *S. coelicolor*, large amounts of actinorhodin are produced due probably to gene dosage and repressor inactivation. Sequencing of this 34 kb fragment could yield information regarding the structure and regulatory elements involved in antibiotic synthesis in streptomycetes[16, 17].

Actinorhodin, granaticin and medermycin are related antibiotics. By introducing actinorhodin genes in a granaticin producer, novel pigments have been produced. When medermycin producer was the host for various actinorhodin gene fragments, new compounds were obtained[23]. One hybrid antibiotic called meder-rhodin A was produced. Microbial biosynthesis of this hybrid antibiotic, by medermycin producing host which harbors genomic fragments of actinorhodin pathway, from *S. coelicolor* is the beginning of a whole new technology. Tools are now available for cloning many antibiotic-modifying enzymes. Putting gene fragments for only a part of the pathway yields mederrhodin. When complete actinorhodin pathway on a 34 kb fragment is

transferred to medermycin producer, hybrid molecules are not produced since enzymes appear to prefer their own natural substrates.

Antibiotic producers possess mechanisms of resistance to the antibiotics that they produce[1, 24]. Kanamycin acetyltransferase gene was cloned into a multicopy plasmid and then introduced into *S. kanamyceticus*, the kanamycin producer, which increased the synthesis of kanamycin 10 times. Genes for biosynthesis of and resistance to antibiotics are clustered. In this way biosynthetic genes have also been selected by selecting for genomic fragments determining resistance to an antibiotic. Cosmids of a herbicide (biolophos)-producing organisms were screened for resistance gene. All the nine steps involved in biosynthesis of biolophos and resistance gene were in one cluster. A 17 kb cloned DNA piece involved in the methylenomycin biosynthesis contains at least three transcription units and may include a regulatory gene and a gene for methylenomycin resistance.

The DNA fragments that promote transcription in *E. coli* also function as promoters in *S. lividans*[25]. Promoter-active fragments isolated in *S. lividans* function poorly in *E. coli*. Analysis of *Streptomyces* promoters will allow the identification of structural features necessary for transcription of *Streptomyces* genes. G. R. Janssen and M. Bibb (John Junes Institute, Norwich, England) constructed plasmid vectors in *S. lividans* for the selection of DNA fragments that promote transcription of either a promoter-less chloramphenicol acetyltransferase or kanamycin phosphotransferase gene. Transcriptional readthrough into the phosphotransferase gene by vector promoters is minimized by the upstream placement of a transcriptional terminator from phage fd. The DNA sequences upstream of structural genes of streptomyces do not show extensive homology to the consensus promoter sequences of *E. coli* or *Bacillus*.

B. subtilis veg promoter has been used to express genes in *Streptomyces*. Both the *E. coli* β-galactosidase and hygromycin phosphotransferase genes are expressed in *S. ambofaciens* when the *veg* promoter and ribosome binding sites are located immediately upstream from the structural gene (Fayerman, Eli Lilly & Co., Indianapolis). Hygromycin phosphotransferase was synthesized at the beginning of stationary phase when controlled by the *B. subtilis* veg promoter. A DNA sequence similar to *Bacillus* veg promoter and to ribosome binding site has been chemically synthesized. This chemically synthesized promoter has a synthetic Hind III site for diagnostic purpose. *In vitro* mutagenesis of this promoter may be used to understand rules of transcription in *Streptomyces*.

A 2.6 kb DNA sequence of *S. coelicolor* A3(2) may function as a transposable element. This DNA does not have homology with known plasmids of *S. coelicolor*, namely, SCP1, SCP2, SLP1 and SLP4 but exists at copy number of less than 0.2 per chromosome. Two linear copies of the 2.6 kb sequence per host genome are also present in *S. coelicolor*, integrated into the chromosome. Despite the absence of homology between *S. lividans* 66 DNA and the 2.6 kb sequence, a ØC31 phage derivative lacking its *attP* site lysogenized *S. lividans* 66 when the 2.6 kb sequence was cloned into it (D. A. Hopwood, John Innes Institute, Norwich, U.K.). K. F. Chater, (John Innes Institute, Norwich, U.K.) has described an insertion element (IS110) of *S. coelicolor* A3(2). This 1.6 kb sequence (LS110) was present in several copies in the *S. coelicolor* genome and absent from the closely related species *S. lividans*[66]. The presence of IS110 in *att*-deleted *ØC31* phages containing a viomycin-resistance marker was used to detect *S. coelicolor* transductants containing the prophage integrated at the various IS110 copies. Two IS110 copies seem to be at well-defined chromosomal locations as shown by the linkage mapping of viomycin resistance.

The integration of the phage vectors containing selectable resistance genes and a variety of unique cloning sites at the preferred chromosomal attachment site allow tests of dominance and complementation of cloned DNA. Alternatively, recombinant phage vectors may integrate in the region homologous with the cloned fragment, resulting in disruption of transcription units. Activation of promoterless genes, such as *lacZ* or the viomycin resistance gene *vph*, in-

corporated into the vector could also occur. Due to Homogenotization, a chromosomally located allele could be transferred to the vector making cloning of flanking sequences and genetic mapping of insertion sequences like cryptic genetic elements possible.

The morphological differentiation of *S. coelicolor* colonies is an apparently complex process whereby vegetative substrate mycelium gives rise to aerial branches which in turn develop chains of spores and concurrently make various secondary products[11]. *S. coelicolor* A(3)2 mutants unable to produce aerial hyphae form colonies which look bald (*bld*). There are four known classes of *bld* mutants. Using a ϕC31 phage vector J. Piret and K. Chater, (John Innes Institute, Norwich, England), isolated two DNA fragments of *S. coelicolor* A(3)2, which restore the capacity to sporulate in two classes of *bld* mutants: *bldA* and *bldB*. These phage derivatives were used to construct partial diploids for the *bld* regions by phage integration and establishment of lysogency in the host chromosome. The *bldA* region consists of at least two genes, one of which is terminally deleted in the cloned insert. A small fraction of mutant lysogens which remain bald release phages and carry *bld* mutations rather than wild type sequences. These colonies could arise through some mechanism of homogenotization. Phages carrying *bldA* mutations could be used to show that *bldA* genes had indeed been cloned and to perform genetic complementation tests and fine structure mapping of *bldA* mutants.

Agarase secreted by *S. coelicolor* A3(2) degrades agar into small oligosaccharides that are utilized as carbon source. The agarase gene is subject to carbon catabolite repression and is inducible by the breakdown products of agar. The gene resides in the 9 o'clock region of the linkage map of *S. coelicolor*. Agarase gene may be a site for integration of the plasmid SCP1 since the resulting NF strains do not produce agarase. The gene has been cloned from genomic DNA of *S. coelicolor* A3(2) into *S. lividans* TK24, employing the vectors pIJ61 and pIJ702. These clones respond normally to catabolite repression, which suggests that the regulatory region of the gene is intact. The coding sequence of the agarase gene is located on a fragment of about 1.8 kb. The extracellular agarase constitutes over half of the total extracellular protein and is about 28–30 kb in size. Intracellular form of agarase is slightly larger, suggesting the existence of a leader peptide analogous to those of other secreted proteins (R. Joseph, G. H. Jones, J. M. Ward, and M. J. Bibb, John Innes Institute, Norwich, U.K.).

GENOME INSTABILITY IN *STREPTOMYCES*

Genomic instability in *Streptomyces* is prevalent and was originally believed to be due to the loss of plasmids. Variants with alteration in aerial mycelium, extracellular enzyme secretion, secondary metabolism, pigmentation, sporulation etc arise frequently and could differ from the wild-type parent by characteristic changes in the genome[3, 15, 16]. In wild-type *S. reticuli* there are three major plasmids of 9, 8.6 and 7.0 megadaltons. These plasmids only share short region of homology. Some of them integrate at various places in the chromosome affecting morphology and secondary metabolism. Melanine negative mutations had deletions of tyrosinase gene as examined by hybridization to probes of PIJ 702. Melanine variants had amplified sequences. Chloramphenicol supersensitive strains of *S. lividans* turned out to be extremely unstable and produced deletions of which 90% were arginine auxotrophs. In some variants, the amplified sequences may represent 5–40% of the total DNA and have a 250 base pair repeat surrounding the amplified DNA. These sequences are not ribosomal, t-RNA or plasmid genes but exist in many copies within the genomes of most strains of *Streptomyces*. Different classes of reiterated sequences exist. There is no understanding of their functions and their possible role in the variability of the *Streptomyces* genome[26].

Independent mutants of *Streptomyces venezuelae* which are unable to convert chorismic acid to *p*-aminophenylalanine, have been isolated. A mutant (Cml-8) accumulated *p*-aminophenylalanine and was probably blocked in the hydroxylation of *p*-aminophenylserine. Another mutant (Cml-2) accumulated three non-chlorinated analogues of chloramphenicol in-

dicating that chlorination of the α-N-acyl group was blocked. The *cml* mutation is consistently mapped to the chromosome between *his*-6 and *strA*6. Seven of the other nine *cml* mutations were mapped to the same general location. This suggests that structural genes controlling chloramphenicol biosynthesis in *S. venezuelae* are located on the chromosome and not on plasmids as suggested by Okanishi[19, 27].

Genetic instability can be due to tandemly reiterated DNA of specific chromosomal regions[3, 16]. Ten independently isolated reiterated sequences from *S. glaucescens* were cloned and analyzed by restriction digests and hybridizations. The amplified sequences occupied up to 45% of the total genomic DNA and ranged in size from 2.9–35 kb per single unit. Cross-hybridization between the cloned sequences and hybridizations to total DNA of the wild-type DNA of *S. glaucescens* revealed that some reiterations arise from single copy, others from low-copy amplified sequences. All reiterated sequences cloned could be allocated to two regions of the *S. glaucescens* genome. Furthermore, some amplifications were accompanied by adjacent deletions. High frequency mutations leading to streptomycin sensitivity and hydroxystreptomycin non-production were identified as large genomic deletions comprising the structural gene for a 6-hydroxystreptomycin phosphotransferase and its flanking sequences. Similarly, mutations affecting tyrosinase production were identified as either structural gene deletions or as mutations involved in the regulation of the expression of the intact structural gene[31].

A 10.5 kb DNA sequence is amplified and tandemly repeated in the genome of *S. fradiae* JS85[28]. A genomic library of DNA from (*S. fradiae* T774), the parent of JS85, before amplification, was screened by plaque hybridization to identify phage clones containing the unamplified 10.5 kb DNA sequence. Recombinant phages with DNA homology to the amplified DNA sequence were selected. A composite restriction map of the amplifiable unit of DNA plus adjacent chromosome was constructed. Detailed restriction mapping identified a 2.2 kb direct repeat at the ends of the amplifiable unit of DNA. A minimum of four copies of the 2.2 kb direct repeat sequence have been identified in the present *S. fradiae* T774.

C. A. Omer and S. N. Cohen[29] have examined the mechanism for the site-specification excision and integration of the SLP1 element of *S. coelicolor* which has implications for the formation of autonomous SLP1 plasmids. SLP1, a genetic element in *S. coelicolor* A3(2) chromosome, is conjugated to *S. lividans*. Integrated from of SLP1 ($SLP1^{int}$) is a 17 kb DNA element. After excision from the *S. coelicolor* chromosome, it exists transiently as a plasmid during transfer to a *S. lividans* SLP1-strain. This transiently existing plasmid directs its site-specific integration into the *S. lividans* chromosome. This unique locus of integration in *S. lividans* corresponds to the original chromosomal location of $SLP1^{int}$ in *S. coelicolor*. Plasmids containing the complete 17 kb SLP1 sequence were constructed by *in vitro* circularization of restriction endonuclease generated DNA fragments of chromosomal DNA carrying a tandem duplication of $SLP1^{int}$ and cloning in the *E. coli* plasmid pACYC177 of a 17 kb *Bam*HI restriction fragment of the complete $SLP1^{int}$ sequence. These plasmids were transformed into *S. lividans* $SLP1^-$. The SLP1 sequence integrated at the same site in the chromosomes of *S. lividans* $SLP1^-$ as when the $SLP1^{int}$ sequence is transferred by mating. Additional DNA is inserted into the plasmid SLP1 integrated into the *S. lividans* chromosome along with the complete SLP1 DNA sequence.

Autonomous SLP1 plasmids generated by mating *S. coelicolor* with *S. lividans* are structural subsets of the transiently existing 17 kb complete SLP1 plasmid. The largest SLP1 plasmid isolated by matings, SLP1.2, retains the 1.72 kb *Pst* I fragment that contains the attachment site for SLP1 but has deleted another region required for integration. The smaller SLP1 plasmids have lost both the attachment site and the region deleted in SLP1.2. An SLP1 plasmid has been constructed in which only the 1.72 kb *Pst* I fragment which contains the attachment site and an adjacent 0.4 kb *Pst* I fragment are deleted. This constructed plasmid does not integrate into the chromosome.

YEAST AND FUNGI

Industrial yeast strains are polyploids or aneuploids, do not possess a mating type, exhibit a low degree of sporulation and yield spores of low viability which makes genetic analyses difficult. Industrial strains are unstable, resistant to ploidy breakdown, cross-breeding and mutational forces. Brewing strains, however, may be manipulated by mutation, hybridization, spheroplast fusion and liposome-mediated transformation. Benomyl, a commercial fungicide reported to induce mitotic chromosome loss, permits recovery of recessive auxotrophic markers indigenous to these strains. The resulting strains should be: 1) rapid fermenters, 2) capable of fermenting wort dextrin, 3) ethanol tolerant, 4) osmotolerant, and 5) capable of utilizing maltose in the presence of glucose, 6) lack undesirable fermentation off-flavours, and 7) possess killer activity.

Brewing yeasts utilize sucrose, glucose, fructose, maltose and maltotriose. Unfermented maltotetrose and larger dextrin molecules can be metabolised by cloning and expressing a glucoamylase gene in brewing yeast. This could allow production of low calorie beer. Glucoamylase gene of *Saccharomyces diastaticus* has been cloned and transferred into both *S. cerevisiae S. pombe*[30]. However *S. pombe* cells secreted active glucoamylase of *S. diastaticus* suggesting that a common feature exists in the protein secretion mechanisms of these two yeasts[31]. However, thermolabile glucoamylase of *Schwanniomyces castelli* may be more desirable in beers instead of the thermostable enzyme of *S. diastaticus*. Up to 15% level of alcohol has no effect on glucoamylase activity.

The yeast *Saccharomyces cerevisiae* can synthesize, process, secrete and assemble eukaryotic proteins[2, 32]. A. Hinnen and coworkers (Ciba-Geigy A. G. Basel, Switzerland) have expressed in yeast, the gene for tissue-type plasminogen activator from the promoter for repressible acid phosphatase. However, K. M. Egan and G. A. Bitter, (Amgen), have constructed generalized expression vectors utilizing the glyceraldehyde 3-phosphate dehydrogenase gene promoter which incorporate the entire yeast 2μ plasmid. These vectors retain the REP1 and REP2 and REP3 locus so that plasmids are stably maintained in CIR° yeast host, and amplify to high-copy number. A completely synthetic interferon-Y gene utilizing optimal yeast codons was expressed from these vectors as 10% of total cell protein. In yeast the 5'-end of the hepatitis B surface antigen gene was replaced with a chemically synthesized fragment. This restored the native glyceraldehyde 3-phosphate dehydrogenase gene untranslated leader resulting in utilization of optimal yeast codons for the first 32 amino acids. This modification elevated expression of hepatitis B surface antigen gene 15-fold yielding hepatitis antigen 2% of total yeast cell protein.

Yeast cells use a mechanism of protein localization very similar to mammalian cells. When the secretion is blocked by mutation, it leads to a block in plasma membrane assembly which is associated with secretion. Three organelles characteristic of secretion are Golgi bodies, endoplasmic reticulum, and secretory vesicles. In yeast, invertase is synthesized and secreted in three minutes. Maturation of invertase in yeast involves addition of a polysaccharide and is a compartmentalized process. Oligosaccharide is first synthesized in endoplasmic reticulum and then linked to N terminal end of invertase in Golgi bodies. When the secretion is blocked by mutation of Golgi bodies, heterogeneous invertase of low mobility accumulates. Non-glycosylated invertase occurs in cytoplasm and does not participate in the process. Conversion of the alanine to valine codon in signal peptide of invertase reduces the frequency of export. A second mutation in structural gene of invertase converts threonine to isoleucine and may affect conformation in such a way that invertase is not properly packaged in Golgi bodies for transport. Retention of the signal peptide affects packaging in Golgi bodies for transport. Cellobiose hydrolase I enzyme of *Trichoderma* is highly glycosylated and secreted in yeast while the other enzyme (endogluconase) is glycosylated to a lesser degree.

Aspergillus has two glucosamylases which differ by 10,000 in molecular weight. Gluco-

amylases are abundantly expressed in *Aspergillus*, forming up to 4% of total cell protein. Differential hybridization of c-DNA constructed from starch-grown cells versus xylose grown *Aspergillus* was used to isolate glucoamylase gene.

The extracellular glucoamylase from *Aspergillus awamori* yields glucose by hydrolysis of starch at the α-1-4 and α-1-6 linkages. The structure and sequence of the *A. awamori* glucoamylase gene, including features of the 5'-promoter region and structure of the four small introns located within the glucoamylase gene appear homologous to several chracterized yeast genes. However, glucoamylase protein or mRNA was not synthesized when Yep vectors carrying the glucoamylase structural gene and considerable 5'- and 3'-flanking sequence were introduced into strains of yeast. Yeast may not recognize the *Aspergillus* enhancer or promoter. Oligonucleotide directed mutagenesis and partial cDNA clones of *A. awamori* glucoamylase to delete the four introns were used. Introduction of a yeast promoter, *ENO1*, at the 5'-end of the gene gave expression of the glucoamylase gene in yeast. The glucoamylase produced is 1% of yeast protein and secreted to the extracellular medium after glycosylation, almost identical to the enzyme in *Aspergillus*[33].

A transformation system has been developed for *C. acremonium* which is used for commercial production of cephalosporin C. Plasmid pIT221 was constructed from pBR322, which allow replication and selection in *E. coli*, an autonomous replication sequence (ARS) from *C. acremonium*, which functions in yeast and a hybrid antibiotic resistance marker. The hybrid gene was constructed by splicing the promoter region of the phosphoglycerokinase (PGK) gene from *S. cerevisiae* to the protein-coding region of a hybromycinphosphotransferase (HPT) gene from plasmid pKC203 *E. coli* ATCC 31912.

When protoplasts of *C. acremonium* were exposed to pIT221 DNA, hygromycin-resistant transformants were regenerated on selective medium. The *In vitro* assays for hygromycin phosphotransferase in transformants derived from *C. acremonium* ATCC 11550 confirmed the transformation events. Restriction digestion of total DNA from transformant and Southern hybridization of the resulting DNA fragments indicated multiple inserts of pIT221 DNA in the DNA of the fungal transformant. Transformants produced CPC in a medium lacking hygromycin B. All cells sampled at harvest were resistant to hygromycin B (personal communication: S. W. Queener, T. D. Ingolia, P. L. Skatrud; J. L. Chapman, K. R. Kaster, Eli Lilly and Co., Indianapolis, Ind. U.S.A.).

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ANNOUNCEMENT

WORKSHOP ON CONSERVATION BIOLOGY

The Workshop on Conservation Biology will be held during February 23 to 9 March 1986 at the Indian Institute of Science, Bangalore.

This workshop will draw on principles from a number of areas of modern biology such as ecology, biogeography, systematics and population genetics, to generate prescriptions for conservation of biological diversity. An exposition of the fundamental concepts will be followed by a consideration of conservation of various biological taxa in different geographical regions of the Indian subcontinent. It will also address itself to the problems of conservation in the context of the socio-economic realities on the Indian subcontinent.

Candidates with M.Sc. or Ph.D. degree in Life sciences/Agricultural sciences/Working in Colleges, universities, research organisations, Botanical and Zoological surveys of India or with forest/wildlife departments or departments of Environment are eligible to apply. The total number of seats is 35. Some financial support towards travel board and lodging could be made available to the participants.

The applications with biodata and reasons for attending this workshop along with names of two referees should reach Prof. Madhav Gadgil, Convener, Centre for Ecological Sciences, Indian Institute of Science, Bangalore 560012, on or before **31st December 1985**