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1. The genetics of self-incompatibility systems

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Self-incompatibility (SI) is very widespread in flowering plants and in a majority of cases is controlled by a single locus with many alleles. The two main SI systems are gametophytic, in which the compatibility of the pollen is determined by the single S-allele it carries, and sporophytic, in which the phenotype of the pollen is determined by the diploid genotype of the male parent. The genetics and other features of these systems will be described with special reference to *Nicotiana* (gametophytic) and *Brassica* (sporophytic).

2. The physiology and histology of pollen-stigma interactions

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The techniques of molecular biology are only now beginning to point to mechanisms by which self-incompatibility systems may operate. It is likely that the molecules involved in these interactions were derived from molecular species which were already performing either a regulatory or architectural function in the pollen stigma interaction. Investigation into the operation of self-incompatibility systems is hampered by the fact that *in vitro* experiments are generally only applicable to plants in which the compatibility of the pollen is under gametophytic control. The regulation of hydration plays such a central role in the operation of most sporophytically-controlled self-incompatibility systems that the principle interactions are almost impossible to duplicate *in vitro*.

The mode of operation of the sporophytically-controlled self-incompatibility system found in the *Brassicaceae* is discussed in detail. Evidence is presented that the molecules involved in self-incompatibility are derived from a large family of compounds which otherwise play a part in regulating hydration of the pollen grain. Surprisingly, self-incompatibility in these plants appears to be under very dynamic control both in the stigmatic papilla cell and within the pollen grain itself. This extraordinary fine regulation of the out-crossing system of these species must make them particularly responsive to changes both within the plant and in the environment that surrounds it.

3. Molecular aspects of self-incompatibility

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The interacting partners during fertilization in higher plants are pollen grains and the female pistil. If mating is compatible, pollen produces a tube which grows through the pistil to the embryo sac. In many plant families, inbreeding is prevented by rejection of pollen tubes after they grow some distance down the style. Rejection is controlled by the product of the *S*-gene, which has multiple alleles, S_1, S_2, S_3 and S_4. We are investigating several aspects of self-incompatibility:

(1) *Nature of the S-gene:* We have isolated cDNA clones encoding the putative S_2- and S_3-allele products. Overall, the sequences are approximately 70 per cent homologous at the nucleic acid level and 80 per cent homologous at the amino acid level. The sequences are punctuated with three highly variable regions which encode hydrophylic

amino acids which are predicted to be on the surface of the protein. Southern analysis of *N. alata* genomic DNA using the S_2 and S_3 cDNAs as probes indicates:

- the gene is restricted to a single locus;
- the gene is present in low copy number (probably single copy);
- characteristic restriction fragment length polymorphisms for the different *S*-alleles.

An S_2-specific antibody has been raised using a synthetic peptide that corresponds to one of the variable hydrophilic regions in the *S*-associated molecules. Using electron-immunocytochemical techniques the antibody has been shown to bind specifically to the intercellular fluid of the transmitting tissue of the S_2 styles, that is, the site of the incompatibility reaction.

(2) *Nature of other style components:* Arabinogalactan-proteins are major components of the extracellular mucilage of the female sexual tissues. These proteoglycans are developmentally regulated and are secreted in increased amounts in the stigma and ovary in response to pollination.

(3) *Structure of pollen tube walls:* Pollen tube walls of *N. alata* have three major components: a $(1\rightarrow3)$-linked glucan, a $(1\rightarrow4)$-linked glucan and a $(1\rightarrow5)$-linked arabinan. The components are organized into two layers with the inner zone containing the $(1\rightarrow3)$-β-glucan demonstrated by immunocytochemistry using an antibody to laminaribiose (Glcp$1\rightarrow3\beta$Glcp) and the outer zone containing arabinan, demonstrated using an anti-arabinosyl antibody.

4. The self-incompatibility genes of *Brassica*: Expression and structure

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The self-incompatibility (S) locus of the cruciferous plant *Brassica oleracea* controls the interaction of male gametic cells with the stigma in the early stages of the life cycle. We show that S sequences are expressed in two tissues of the flower. *In situ* hybridization experiments localized the S transcripts to the papillar cells of the stigma surface, and Northern analysis revealed that S transcripts are expressed during pollen maturation in post-meiotic anthers. A number of the multiple S-homologous sequences derived from the S locus of *Brassica* and from the related self-fertilizing *Arabidopsis* have been cloned and analyzed. Only one of these copies is apparently expressed in *Brassica* stigmas and is intronless.

5. The population genetics of the self-incompatibility polymorphisms

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The two chief questions that we wish to ask about the properties of the self-incompatibility polymorphisms concern the factors which determine, respectively, the number and frequency of alleles in a population. Because these polymorphisms are maintained by gene frequency dependent selection, the number of alleles that could be maintained in a population of a species with a homomorphic system of self-incompatibility is, theoretically, very large. Dynamic and, perhaps, molecular constraints, however, will impose limits on this number. The results from analyses of the number of alleles in natural populations of a number of species will be discussed in the context of these theoretical considerations.

While the frequencies of S-alleles in natural populations of species with the homomorphic, sporophytic system found in *Brassica* are expected to be unequal, the frequencies of recessive being higher than those of dominant alleles, these frequencies are expected to be approximately equal in populations of species that possess a gametophytic system of self-incompatibility. The frequencies of the incompatibility alleles in four out of five natural populations of species with gametophytic systems that have been analysed in sufficient detail, however, have turned out to be significantly unequal. Some of the possible causes of these inequalities will be discussed.

6. Introductory remarks: Growth, and the switch into antibiotic productions in *Streptomyces*

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The advent of recombinant DNA techniques for *Streptomyces* has resulted in the cloning of numerous gene clusters encoding antibiotic pathways and, more recently, of some genes

involved in the development process. This work now provides the tools to investigate expression of developmentally-regulated genes at the molecular level. It will result ultimately in an understanding of how control of streptomycete gene expression is integrated. However, a complete description of antibiotic production will also require an insight into the metabolic control of the enzymes of the pathways.

With a few exceptions, primary metabolism has been given little attention in the last fifteen years. Primary metabolism is responsible for providing the many precursors of growth. But, when the growth phase is over, it is also responsible for provision of large quantities of the few precursors necessary for antibiotic biosynthesis. An understanding of the expression and subsequent metabolic control of these genes and their gene-products is a necessary step to an overall description of the streptomycete life cycle. It will also be useful in realising more fully the genetic potential of the genus to provide products of use to us.

7. Aspects of the regulation of the glycerol utilisation operon of *Streptomyces coelicolor* A3(2)

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The glycerol utilisation (*gyl*) operon of *Streptomyces coelicolor* A3(2) is subject to glycerol induction and glucose repression. The three genes of the operon, *gylA* (presumptively encoding glycerol kinase), *gylB* (encoding *sn*-glycerol-3-phosphate dehydrogenase) and *gylX* (of undefined but non-essential function) are transcribed consecutively to give a 5·4 kb mRNA. A smaller discrete *gyl* mRNA species of 4·3 kb is also produced, and arises either from endonucleolytic processing between *gylB* and *gylX*, or from transcription termination at this point (Smith and Chater, *Mol. Gen. Genet.*, in press, 1987). *gyl* operon transcription starts from either of two promoters which are separated by 50 bp. Although both promoters are glycerol-inducible and glucose-repressible, *gyl*P1 (the upstream promoter) is more sensitive to both forms of regulation. Preliminary studies implicate a transcription anti-termination mechanism in the control of *gyl* operon expression. This, and the possible biological significance of the tandem *gyl* promoters, will be discussed.

A 0·9 kb transcription unit, *gylR* (formerly designed the *0·9 kb* gene), is located immediately upstream of the *gyl* operon. *gylR* is transcribed from a single promoter which is subject to glycerol induction but not glucose repression. Genetic studies and comparative protein sequence analysis suggest that *gylR* encodes a regulatory protein which activates *gyl* operon transcription.

8. RNA polymerase heterogeneity and the transcription of the agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2)

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The *dagA* gene of *S. coelicolor* encodes an extracellular enzyme which is involved in the catabolism of agar. Analysis of the transcription of the *dagA* gene identified four promoters which initiate transcription approximately 32, 77, 125 and 200 bp upstream of the coding sequence. Using a combination of FPLC gel filtration and anion exchange chromatography, three different RNA polymerase holoenzymes were separated, each of which transcribes from only one of the *dagA* promoters. Holoenzyme reconstitution experiments identified the sigma factors responsible for recognition of two of the promoters. The previously characterised $E\sigma^{49}$ transcribes from the *dagA* p3 promoter while a novel species, $E\sigma^{28}$, recognises the *dagA* p2 promoter. Circumstantial evidence suggests that the third holoenzyme, which transcribes from the *dagA* p4 promoter, is the previously characterised $E\sigma^{35}$.

9. Nitrogen regulation in *Streptomyces coelicolor*

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The regulation of glutamine synthetase (*glnA*), a key enzyme in nitrogen assimilation, is being used as a model system for investigating global nitrogen regulatory mechanisms in *Streptomyces coelicolor*. Two approaches have been taken. First, the gene encoding the *glnA* structural protein was cloned.

The coding region of *glnA* was identified by DNA sequencing and N-terminal sequencing of the purified *glnA* protein. Two *glnA* promoters were identified by primer extension experiments: an upstream constitutive promoter, and a downstream nitrogen-regulated promoter. To identify the nucleotide sequences required for nitrogen regulated transcription, various promoter fusions to the galactokinase structural gene (*galK*) were constructed. The expression of these *glnA-galK* fusions in *S. coelicolor* cells grown in media containing excess and limiting nitrogen is being examined.

A genetic approach is also being used to study nitrogen regulation of glutamine synthetase. Several glutamine-requiring *S. coelicolor* mutants have been isolated. One class of mutants has been shown to be defective in a positive regulatory factor required for transcription of the nitrogen-regulated *glnA* promoter. *S. coelicolor* DNA complementing these Class 1 GlnA regulatory mutants has been isolated and is being characterized.

Other *Streptomyces* genes are being examined for nitrogen regulation. Since significant nucleotide sequence homology exists between the nitrogen-regulated *glnA* promoter and the *tsrpl* promoter of the thiostrepton resistance gene, the expression of the *tsrpl* promoter in *S. coelicolor* cells grown with either excess or limiting nitrogen is being determined. In addition, the expression of enzymes likely to be subject to nitrogen limitation is being examined. Preliminary experiments suggest that the expression of urease in *S. coelicolor* is nitrogen-regulated, and that this expression is significantly reduced in the Class I GlnA regulatory mutants.

10. Organization and regulation of the genes for biosynthesis of the herbicide antibiotic bialaphos in *Streptomyces hygroscopicus* and *S. viridochromogenes*

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11. Transcriptional and translational control elements in *Streptomyces* differentiation

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During *Streptomyces coelicolor* colony development, an initial vegetative mycelium gives rise to aerial hyphae which subsequently form spore chains. This morphological differentiation is accompanied by the onset of antibiotic production. Mutations that interfere with aerial mycelium formation define *bld* genes, some of which are also needed for antibiotic production. One such gene, *bldA*, encodes a tRNA recognising the leucine codon UUA, which is very rare in [G+C]-rich *Streptomyces* DNA. This gene is dispensable for normal vegetative growth, but is required for the expression of cloned TTA-codon-containing genes. The TTA codon therefore appears to be absent from all genes essential for vegetative growth. A second class of developmental genes, the *whi* genes, are defined by mutations that prevent aerial hyphae from developing into spore chains. One of the earliest requirements during sporulation appears to be for the *whiG* gene product. The *whiG* DNA sequence reveals that it specifies a sigma factor-like protein. The level of abundance of this protein determines the extent of sporulation: multiple copies of *whiG* cause sporulation to occur even in vegetative hyphae. Gene disruption experiments suggest that *whiG* has no important role during normal vegetative growth.

12. Visualizing gene expression in time and space during morphological differentiating in *Streptomyces coelicolor*

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Differentiation in *Streptomyces* involves two spatially separated types of cells: the branching hyphae of the substrate mycelium, which penetrate the substratum upon which the colony feeds, and the upwardly protruding hyphae of the aerial mycelium, which undergo metamorphosis into spores. The luciferase-encoding *luxA* and *luxB* operon of the luminescent marine bacterium *Vibrio harveyi* was used as a promoter probe to visualize

gene expression in differentiating colonies of *Streptomyces coelicolor*. Promoters for developmental genes of several kinds, including *bld2* and *whiG* (studied in collaboration with E. Lawler, C. Mendez and K. Chater), *sapA*, which encodes a spore-associated protein, and two promoters identified in a shot-gun library on the basis of their temporally-delayed induction, gave distinct temporal and spatial patterns of light emission.

In other work, we have identified a 3 kDa polypeptide called SapB that is an abundant component of *S. coelicolor* spores. SapB appears at the time of aerial mycelium formation. Its appearance is impaired, however, in all *bld* mutants tested and in *whiE* and *whiH*, but not in *whiA*, *whiB*, *whiG* mutants. Unusual features of SapB are that it is composed of only nine different amino acids and that it appears to be a glycoprotein.

13. Identification and regulation of genes and promoters involved in transfer of the pIJ101 plasmid

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Genes mediating transfer of the pIJ101 plasmid have been identified and delineated using a series of linker and pUC 19 insertions, and endonuclease-generated pIJ101 DNA fragments that contain individual transfer-related genes have been cloned and characterized functionally. Promoters controlling expression of the *kil* and *kor* genes (K. J. Kendall and S. N. Cohen, *J. Bact.*, *169*, 4177–4183, 1987) within the transfer regions have been studied using a series of transcriptional fusions to bacterial β-galactosidase and luciferase genes. Sequences responsible for promoter activity have been studied by mutational and deletion analysis. The *kil* and *kor* promoters resemble the *E. coli* type promoters (SEPS) found previously in *Streptomyces species*. Between the −10 and −35 regions of the *kilA*, *kilB* and *korB* promoters are extensive dyad symmetries. The nucleotide sequence of the entire plasmid has been determined and open reading frames (ORFS) have been correlated with available genetic data. Two ORFS that encode proteins containing possible helix-turn-helix domains have been observed at the locations of the genetically identified *korA* and *korB* genes. Functional analysis indicates that mutations within one of the helix-turn-helix domains in the *korB* ORF decrease the regulatory activity of the putative *korB* protein.

14. Biology of the *Streptomyces* multi-copy plasmid, pIJ101

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pIJ101 is a 8·9 kb multi-copy plasmid (*ca.* 300 copies) with a wide host-range in *Streptomyces*. pIJ101 is self-transmissible, induces "pock" formation and acts as a fertility factor (*i.e.*, promotes recombination of chromosomal genes).

pIJ101 seems to replicate via a single-stranded intermediate which accumulates in some of the commonly-used DNA vectors derived from this plasmid. The main site for the initiation of second-strand synthesis has been located outside the essential replication region. Plasmids which lack this site cannot co-exist with their parental plasmids which have the site, while pairs of plasmids which either both have, or both lack, the site can be maintained in the same cells at similar copy numbers. pIJ101 derivatives which have the site for second strand synthesis inverted behave like plasmids which lack it altogether except that they readily undergo recombination with wild-type plasmids.

The copy number of the double-stranded form of pIJ101 derivatives depends on the presence or absence of the site for second-strand initiation and on a *trans*-acting element which probably represses initiating of second-strand synthesis. Thus, plasmids lacking the site for second-strand synthesis have a reduced copy number (*ca.* 50–100) while absence of the repressor gene can lead to a copy number of *ca.* 1000 if the site for second-strand synthesis is present in its correct orientation with request to the essential replication region of the plasmid.

15. Deletions and amplifications in Streptomycetes

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Streptomycetes exhibit a remarkable degree of genetic variability. This is usually observed in the

form of genetic instability, *i.e.*, loss of certain phenotypic traits such as aerial mycelium formation, antibiotic synthesis and resistance, or melanin formation. Analyses at the DNA level have shown that loss of a trait is due to deletion of the corresponding structure gene(s) and of neighbouring DNA regions. In the same analyses frequent amplifications of DNA segments directly adjacent to, or in the neighbourhood of, the deletions were observed. Basically the same phenomena have been described for a number of strains. The best analyzed examples are *Streptomyces fradiae*, *S. glaucescens* and *S. lividans*. The extent of deletions and amplifications in the genome of *S. glaucescens* strain ETH22794 will be discussed and the results will be compared to those obtained with *S. fradiae* and *S. lividans*.

Two unstable traits have been investigated in *S. glaucescens* in some detail. These are melanin formation and streptomycin resistance. In most non-melanogenic mutant strains the structural gene for tyrosinase, *melC*, is deleted (Hintermann *et al.*, *Mol. Gen. Genet.*, *200*, 422–432, 1985) and streptomycin-sensitive derivatives exhibit loss of *strC*, the structural gene for a streptomycin-6-phosphotransferase (Hintermann *et al.*, *Mol. Gen. Genet.*, *196*, 513–520, 1984) and of some genes involved in (hydroxy) streptomycin biosynthesis (Vögtli and Hütter, *Mol. Gen. Genet.*, *208*, 195–203, 1987). We could show that in *strSmelC* double mutant strains at least 700 kb of chromosomal DNA are deleted, comprising the *strS* and *melC* areas, which are 350 kb apart. *strSmelC*$^+$ single mutant strains contain deletions of around 400 kb, spanning the *strS* area, and ending between *strS* and *melC*. Most deletions have one end point in common, but span variable lengths of the chromosome. The longer deletions observed approach the area of DNA amplifications or extend to the end of the amplified DNA elements (Hasegawa *et al.*, *Mol. Gen. Genet.*, *200*, 375–384, 1985). The units of DNA amplifications (ADS) are present in single copies in the parent genome, and in no case was the same amplification observed twice.

This is in contrast to the observations with amplifications in *S. fradiae* and *S. lividans*, where duplications of ADS, with long direct repeats at the ends, are present in the wild type genome, permitting the same amplifications to occur repeatably and at high frequency (Altenbuchner and Cullum, *Mol. Gen. Genet.*, *201*, 192–197, 1985; Fishman *et al.*, *J. Bacteriol.*, *161*, 191–206, 1985). Also in *S. lividans* deletions occur immediately adjacent to the amplifications (Dyson and Schrempf, *J. Bacteriol.*, *169*, 4796–4803, 1987).

Models for deletion formation and for amplification will be discussed.

16. Wheat genetics—An overview

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Wheat genetics has benefitted greatly from the development of precisely defined genetic stocks. These have been created through the application of cytogenetical methods and the IPSR is fortunate in being the leading laboratory for the collection of such stocks and their exploitation in genetic studies.

Their use in analysis is not restricted to the identification and chromosomal location of genes for qualitative characters, but extends to genes affecting quantitative characters of economic importance. Many of these latter genes have been recognised and their effects estimated.

Since wheat is a polyploid it is also possible to study the activities of genes by varying their dose. In combination with location studies this is a valuable approach to understanding the processes controlled by particular genes. Defined wheat stocks are increasingly being used in molecular biology; in the development of genetic maps using molecular probes; in the study of gene structure; and hopefully in the future for the isolation of important genes.

17. RFLPs as genetic markers in wheat

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Although wheat (*Triticum aestivum*) is an important crop and has long been the object of genetic research, useful markers are sparse and the genetic map is not highly developed. This is in large part due to the polyploid ($2n = 6x = 42$) nature of wheat where recessive alleles are often masked by compensating genes in other genomes. Restriction fragment length polymorphisms (RFLPs) offer the prospect of finding many codominant marker "loci" which have few pleiotropic effects on agronomic characters, and thus are of value for use in conventional breeding as well as research.

Our progress towards the goal of mapping the genome at more than 400 loci (at ~10 cM intervals)

will be discussed, with particular reference to screening a small cDNA library, and to the variation identified by a β-amylase probe at the RFLP level in comparison to variation detected by isoelectric focussing of grain β-amylase isozymes. The applications of wheat RFLPs and their mapped locations are extensive and include gene tagging in early generation breeding screens, uses in the characterization of useful genes from alien sources and their introgression into wheat by enhancement of cytological analysis, as well as potential starting points for the isolation of genes of agronomic importance.

18. Long range mapping in peas

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Pulsed field gradient gel electrophoresis and related techniques afford an opportunity to study genome organization at a level intermediate between that accessible to conventional restriction enzyme mapping and genetic analysis. If the potential of these techniques is to be realized in the analysis of higher plant genomes, then suitable DNA extraction procedures need to be developed. We have begun to investigate techniques which may be appropriate for the isolation of high molecular weight DNA from plant cells. Although these techniques are not yet optimized, we can report some successful results obtained using the technique of field inversion gel electrophoresis.

19. Biochemical basis of the differences between round and wrinkled pea seeds

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The *r* (rugosus) locus of peas determines whether the pea seed is round or wrinkled. It also has profound effects on the composition of the seed, particularly on its starch content. Round seeds (*RR*, *Rr*) have high levels of a "normal" starch whereas wrinkled peas (*rr*) have lower levels of a starch with an unusually high amylose (unbranched starch) content. These differences in composition suggest that the primary effect of the *r* locus is on a starch branching enzyme. Changes in the activity of this enzyme during development of round and wrinkled seeds, and the properties of the enzyme purified from these seeds, indicate that one isoform of the enzyme is present in round seeds but missing from wrinkled seeds. This difference may explain all of the effects of the *r* locus on seed development.

20. The molecular basis of variation in genes encoding wheat seed storage proteins

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The major seed storage proteins of wheat are specified by multigene families at several loci. All the genes appear to have evolved from common ancestral sequences. Extensive variation within and between loci has been characterised by electrophoresis of protein products as well as by sequencing of cDNA and chromosomal copies of the genes. The molecular basis of this variation will be described together with the processes responsible for it. Variation in the regions which determine the seed-specific expression of the genes will be similarly discussed. These regions have been defined by their ability to determine endosperm-specific expression of a reporter gene in transgenic tobacco plants.

21. *Agrobacterium*-mediated transfer of geminivirus DNA to cereals

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Agrobacterium has been in use in conjunction with cereal-infecting geminivirus DNA to demonstrate that it can transfer DNA to various members of the *Graminae*.

Agrobacterium tumefaciens and *Agrobacterium rhizogenes* are pathogens of a wide range of dicotyledenous plants which cause hormone independent tumour or hairy root proliferation respectively. The cause of this abnormal cell proliferation

is the transfer of a portion of the Ti plasmid DNA (called T-DNA) from bacterium to the plant cell genome. Several of the genes in the T-DNA code for enzymes involved in auxin and cytokinin synthesis and are responsible for the hyperplasias described above. Transformation of plant cells employs disarmed Ti plasmids which have their oncogenic functions removed. However, until recently, it appeared that the *Graminae*, which include all the major cereal crops, were refractory to infection by *Agrobacterium*.

Germiniviruses are a group of plant viruses which are characterized by the possession of twinned quasi-isometric particles and genomes of circular single-standard DNA. Those which infect the cereals, such as Maize Streak Virus and Wheat Dwarf Virus, are not mechanically transmissible. The insertion of geminivirus DNA into a disarmed Ti vector and the inoculation of the plasmid into several graminaecous species *via Agrobacterium* (termed agroinfection) results in the appearance of typical virus symptoms several weeks later. No tumours were observed on infected plants and no T-DNA has been detected, but nevertheless these data show that *Agrobacterium* can transfer DNA to cereal cells overcoming a major obstacle in the development of a transformation system for cereals.

22. Transcriptional regulation of genes during potato tuberization

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The development of potato tubers represents a complex redirection of the growth of the stem to a non-photosynthetic plagiotropic stolon. We have set out to study the differential expression of genes during tuber formation using gene fusions and transformation. The two necessary components of this analytical system, a sensitive and versatile reporter gene, GUS, and an efficient and rapid transformation method for potatoes, have been developed recently in this laboratory. We have made fusions of the GUS gene and the putative transcriptional regulatory sequences of a patatin gene isolated from Maris Piper. The patatin multigene family encodes a diverse group of 40 kd glycoproteins that comprise approximately 40 per cent of tuber protein. As patatin is not normally found in leaves, roots or stems of potatoes but is induced over 1000 fold in tubers, it is a suitable marker for tuber differentiation. Patatin induction can occur in the absence of tuber formation, but patatin induction always occurs during tuberisation. This indicates that tuberisation is a multistage interdependent process in which morphological changes can be uncoupled from biochemical changes such as patatin accumulation.

In order to analyse the activity of the patatin promoter various lengths of 5′ non-coding patatin sequences were linked to the GUS gene and introduced into potato using *Agrobacterium* transforming vectors. An *in vitro* tuberisation system was used to induce patatin in cuttings of transformants. Analysis of over 10 transformants for each promoter construction gave rise to the following picture:

(1) the first 360 bp of the promoter is sufficient for a low level of induction.
(2) a 650 bp promoter stimulates a higher level of GUS activity in both induced and non-induced explants.
(3) a 950 bp promoter expresses a similar higher level only in induced explants. This promoter fragment may contain a tissue or induction specific "silencer".
(4) longer promoter fragments appeared to give higher levels of induced GUS activity. After 14 days GUS levels were approximately 1000 fold higher in induced cuttings containing a 2500 bp promoter. Histochemical examination of tubers induced on plants containing a 2500 bp promoter shows that patatin transcription occurs early in tuber formation in the stem. As stolon growth and tuberisation progresses, cells expressing patatin become more widespread until in a mature tuber all cells except the dead periderm express patatin.

These preliminary *in vitro* experiments are being reproduced on a much larger scale in a field trial involving nearly 2500 transformed plants, in collaboration with colleagues at Unilever.

Future work will involve a more detailed analysis of the patatin promoter using hybrid promoter constructions and DNA-protein interaction experiments. In addition, a study of day-length induction will be conducted in appropriate varieties. Finally, a study of the role of the putative transit peptide of patatin in protein storage and glycosylation is in progress.

23. Development of systems for study and use of transposable elements in heterologous species

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Transposable elements have been used as molecular tags to clone genetically-marked loci in a number of eucaryotic species with well-characterised transposon systems, such as *Drosophila*, *Zea mays* and *Antirrhinum*. The advantage of this cloning strategy is that it offers a means of identifying a gene at the molecular level simply by its phenotype, without a detailed knowledge of the protein that it encodes. Many genes that are particularly interesting in this respect (for example morphological genes and those that confer resistance to particular pathogens) may be restricted to commercially important species that do not as yet, have well-characterised transposon systems of their own. It has been demonstrated that plant transposons can be introduced into a foreign host and can retain the ability to transpose at high frequency. This offers the possibility of establishing heterologous transposon systems in species of choice for transposon-tagging of particular genes.

We have demonstrated that the *Antirrhinum* transposon Tam3 will transpose in tobacco, showing that it may have some potential in this respect, as it has a number of attributes, such as temperature sensitivity, which are very useful in tagging experiments. However there are also clearly some restraints imposed on Tam3 transposition in tobacco, which in turn shed light on control mechanisms operative in *Antirrhinum*. Thus, transformation offers the possibility for powerful tagging systems in the near future, but also provides an immediate system for detailed analysis of the transposons themselves.

24. Chimeric genes associated with cytoplasmic male sterility

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Cytoplasmic male-sterility, an inability to produce functional pollen, is a maternally transmitted phenotype. Comparative analysis of the mitochondrial and chloroplast DNAs in fertile and sterile plants indicates that it is the mitochondrial genome that specifies the male-sterile phenotype. Molecular studies in two species, *Petunia* and maize, have identified unusual chimeric genes, whose expression correlates directly with the inability to produce functional pollen. The transfer of such genes into fertile tobacco using *Agrobacterium* mediated-transformation is being undertaken to see if male-sterility can be induced.

25. Evolution of plasmid replication and maintenance systems

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The replication of bacterial plasmids which have so far been studied in detail, fall into three main classes. ColE1-like plasmids utilize a processed RNA polymerase transcript as a primer for leading-strand synthesis. pT181- and pC194-like plasmids replicate *via* a rolling circle mode, initiated by the plasmid-encoded Rep protein nicking the circular double stranded molecule. The replication of these plasmids shows considerable homology to that of single stranded DNA phages like ϕX174. Most other plasmids exploit the binding of a Rep protein to direct repeats at the origin to initiate replication.

Plasmid-encoded control over replication is exerted in three general ways. First, *trans*-acting antisense RNA molecules repress preprimer processing or translation of *rep* mRNA. Second, repressor proteins limit *rep* gene expression. Third, *cis*-acting DNA sequences adjacent to the replication origin play a negative role either by titrating Rep protein or by promoting a non-productive complex with the replication origin and the Rep protein.

In addition, many plasmids have acquired mechanisms for one or more additional functions: for active partitioning to daughter cells; for the resolution of multimers; or for lethality to plasmid-free segregant bacteria.

The relationship between functionally related systems on different plasmids and their possible origins in non-plasmid systems will be discussed. For example the *parB* locus of RI is related to an *Escherichia coli* chromosomal gene.

Finally the evolution of the IncP plasmid group will be discussed with respect both to the development of a complex stable inheritance system and to the retention of functionally interchangeable elements despite considerable sequence divergence between different members of the group.

26. Evolution of conjugative transfer systems

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The definition of conjugation as genetic transfer requiring contact between donor and recipient bacteria encompasses a variety of systems. Discussion will focus on conjugation of gram-negative bacteria and, in particular, on the systems encoded by plasmids of the F, B, I, N, and P incompatibility groups. The genetic diversity of these systems, common organisational features, and the evolutionary relationship between conjugative system and incompatibility group will be reviewed.

Each system includes genes affecting cellular interactions (pilus and exclusion determinants) and genes for the processing of the plasmid DNA. Pili can be separated into different morphological classes that promote conjugation in preferred environments. Exclusion genes have evolved to inhibit DNA transfer between cells harbouring closely related plasmids. DNA processing involves nicking at the origin of transfer site, DNA unwinding and transfer of a specific strand to the recipient cell where the complementary strand is synthesised. Multifunctional polypeptides have evolved to promote some of these stages. These polypeptides act analogously to certain bacterial and phage DNA replication proteins and are transmitted selectively to the recipient cell.

27. Plasmid evolution and the release of genetically engineered microbes

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28. The role of transposable elements in the evolution of plasmids

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Transposable elements are discrete DNA sequences that, in general, encode functions that allow them to recombine into other sites on the same or on a different DNA molecule. They can mediate plasmid evolution in several ways. The most obvious change for which these elements are responsible and the primary result of their activity is the addition to DNA molecules of new nucleotide sequences *i.e.*, those that define the element, including genes unconnected with transposition. Secondary consequences, such as deletion or sequence inversion may then follow, as a direct result of further transposition activity or as the result of the host cell's recombination system acting on regions of intra-molecular nucleotide homology. Transposable elements can also mediate fusions between two DNA molecules, either directly, as a consequence of transposition, or indirectly, by providing regions of nucleotide homology on different DNA molecules that can serve as substrates for the host cell's recombination system.

These various activities rearrange DNA sequences, more or less at random; the success of a particular rearrangement is likely to depend on several factors, including the identity of the particular sequences rearranged, functions newly acquired and the utility of the new plasmid to its host. These points will be developed and illustrated.

29. The maintainence of the transposons in bacterial populations: theoretical predictions and experimental observations

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We have developed mathematical models of the population dynamics of transposons in populations of bacteria carrying conjugative plasmids. The results of our analysis of the properties of these models indicate that there are conditions under which replicative (but not conservative) transposons can become established and maintained in bacterial populations by hitchhiking on conjugative plasmids. This can obtain even when these movable elements confer a fitness disadvantage on their host bacterium. The conditions for maintaining these purely "parasitic" trans-

posons are, however, restrictive: (1) the cost of carriage of the transposons has to be low, on the order of the transposition rate; (2) the rate of plasmid turnover (transfer to new recipients) has to be high, in excess of that anticipated for natural populations. Even when these conditions are met, the rate of ascent of a parasitic transposon is of the same order as the transposition rate 10^{-4} or lower per generation.

In an effort to examine the reality of this model and validity this prediction, we studied the population dynamics Tn3 and Tn5 in chemostat and serial transfer populations of *E. coli* K-12 and the plasmid R100-1. We estimated the parameters of this model and followed the fate of Tn3 and Tn5 when low frequencies of *E. coli* carrying these transposons on R100-1 were introduced into populations of *E. coli* carrying this plasmid without the transposon. We interpreted the results of the invasion-when-rare experiments as being consistent with the prediction made from our theoretical analysis, that transposition would contribute little to the ascent of transposons and their maintenance in bacterial populations.

We discuss the implications of these theoretical and experimental results to hypotheses about the evolution and maintenance of bacterial transposons.

30. A theoretical point of view on plasmid evolution

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Many properties of plasmids, like conjugation and surface exclusion, directly affect the dynamics of plasmids in a bacterial population. In which direction such properties will evolve in a plasmid population can be calculated using some of the methods developed in Eukaryote population genetics. For instance, the Evolutionary Stable Strategy (ESS) concept: It is the combination of properties of a certain plasmid stable against invasion by a mutant of that plasmid with a (slightly) other strategy.

Using such methods I have investigated whether a population of plasmids can be invaded by a mutant inducing Surface Exclusion (SE). In this calculation it is assumed that the membrane adaptations, causing SE, decrease the growth rate of the bacterial host. It appears that SE against plasmids of the same incompatibility group is advantageous if the transfer rate is high and the copy number low. Against compatible plasmids it will not be profitable if the excluded plasmid increases the growth rate of the host and the transfer rate is not affected by the presence of the other plasmid.

31. The evolution of catabolic plasmids

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Since 1972 the existence of plasmids encoding the catabolic pathways for a wide range of carbon compounds including aromatic and aliphatic hydrocarbons, xenobiotic pesticides, halogenated compounds and naturally occurring terpenes have been reported. Most of these have been found in members of the genus *Pseudomonas* and the two best studied are involved in toluene/xylene and in naphthalene catabolism (TOL and NAH plasmids respectively).

The evolution of these plasmids will be considered from the point of view of (a) their structural plasticity under changing biochemical selection pressures (b) the relationship between the DNA encoding similar biochemical sequences on different plasmids and (c) the existence on some of relatively stable diverge gene duplications. The hypothesis will be proposed that some of the more complex pathways, as found on the TOL and NAH plasmids, may have evolved from the acquisition of metabolic "modules".

32. Evolutionary relationships among antibiotic resistance determinants

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Antibiotic resistance genes are found, predominantly, in two classes of micro-organisms: antibiotic producers and clinical isolates from humans or animals. The roles of the resistance determinants in the two sources are essentially identical, in that they are required to protect the host organisms from the inhibitory action of exogenous or endogenous antibiotic substances.

At the mechanistic level, the "identity" of antibiotic resistance determinants from the two sources has been confirmed since similar target site modifications, altered transport or detoxification mechanisms are found for the same antibiotic classes.

Although there is little overall similarity between the structures of genes or enzymes responsible for resistance to any given antibiotic or group of antibiotics (nucleic acid hybridisation and antibody cross-reaction studies have proved negative), nevertheless strong "domain" relationships have been identified at the polypeptide level.

The aminoglycoside modifying enzymes have been studied extensively and clear evolutionary relationships can be discerned, particularly among the phosphotransferases of streptomycetes and those of gram-positive and gram-negative bacteria of human origin. How could genes be disseminated among such diverse bacterial species? Although a graduated series of gene transfers among closely related organisms is probable, recent studies have demonstrated direct conjugal transfer of antibiotic resistance plasmids between gram-positive and gram-negative bacteria, and *vice versa*. The selective pressure of antibiotics must have played a role in antibiotic resistance gene dissemination in hospitals, but the role of antibiotics in natural situations is less clear. Some evidence suggests that antibiotic substances may promote conjugal transfer without necessarily exerting a selective function.

Recent analyzes of the amino acid sequences of aminoglycoside phosphotransferases have shown strong similarities with the sequences of mammalian protein kinases. This implies that these enzymes are members of a widely distributed protein family with a common evolutionary origin.

33. Site-specific base modifications in the DNA of *Streptomyces lividans*

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Streptomyces are remarkable micro-organisms for their ability to produce a vast array of secondary metabolites, their complex differentiating growth pattern, and because they exhibit an extremely high spontaneous mutability of certain genes. The latter, termed genetic instability, is characterised by the generation of very large chromosomal DNA deletions which are often accompanied by hundred-fold amplification of specific DNA elements located at one deletion end-point. Genetic recombination causing these gross DNA rearrangements may be stimulated by "micro"-modifications of the *in vivo* DNA structure.

One type of site-specific base modification is present in both the amplified DNA and in other high copy number amplified DNA targets, for instance plasmid molecules, of *Streptomyces lividans* strains. The modification confers alkali and thermo-lability to the sugar-phosphate backbone in one DNA strand at the position of the altered base. It is likely that single-copy DNA sequences are similarly modified. DNA sequence analysis demonstrates that the modification is of either a guanine or cytosine residue located within a 10-nucleotide consensus sequence. The ubiquity of the modifications suggests the involvement of a *trans*-acting factor in their generation. Circumstantial evidence incicates that this factor is a DNA-binding antibiotic.

34. The genetic analysis of a sporulation factor from *Streptomyces coelicolor* A3(2)

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Several mutations of *S. coelicolor* A3(2) have been isolated and characterised, that block the development cycle. One of these mutations appears to have uncovered a pheromone based cell-cell signalling system that operates during spore formation. The strain J830 is a bald (*bld*) mutant that does not produce normal aerial mycelium. All of the bld^+ strains tested, produce a "factor" that induces sporulation in J830.

The factor is produced in agar and liquid media and has been partially purified. None of the other bald mutants appear to respond to any factor.

J830 may have lost the ability to produce the pheromone but can still respond to it. The gene malfunctioning in J830 has been the subject of classical genetic analysis and molecular cloning.

35. Plasmid mediated restriction and modification in *Acinetobacter sp.*

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Evidence will be presented for a novel plasmid mediated restriction and modification system active on broad host range plasmids in an alkane utilising *Acinetobacter sp.* Genetic studies using the transmissable R-factor pAV1 have demonstrated that this system is distinct from the previously described system specified by pAV2 in *Acinetobacter sp.* EBF65/65. Evidence will also be presented indicating that pAV1 is able to escape plasmid mediated restriction by the mobilisation of cryptic plasmids.

36. Evolution of a group of staphylococcal plasmids

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The complete nucleotide sequence of a naturally occurring *Staphylococcus aureus* plasmid, pT48 (from *S. aureus* strain T48), has been determined. The 2475bp plasmid confers inducible resistance to macrolide-lincosamide-streptogramin B (MLS) type antibiotics and shows homology with *S. aureus* plasmids pE194 (MLS^R) and pSN2 (cryptic). It also contains a palA structure homologous so that on *S. aureus* plasmid pT181.

Similar plasmids, some of which confer constitutive MLS resistance, have been described in *S. aureus* and in other Gram-positive organisms. The role of antibiotics such as tylosin in the evolution of constitutive MLS resistance from the inducible phenotype has been noted previously. Selection for partial tylosin resistance has led to the isolation of a variety of mutants of pT48 with highly multimeric forms of DNA. This technique may have mimicked the natural evolution of plasmids with this unusual phenotype. Further analysis of these mutations fit an explanation involving defective termination of a rolling circle type replication mechanism.

Other experiments have demonstrated features, within the pSN2 homologous region essential for the replication and maintenance of pT48. The nucleotide sequence differences between this region and that of pSN2 and similar cryptic plasmids may represent evolutionary divergence in the specificity of a rep/ori interaction.

37. Mobilisation of the multicopy plasmid NTP16 by the F factor

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The small non-conjugative plasmid NTP16 is readily transferred from donor to recipient cell when co-resident in the donor with the conjugative plasmid F. The relatively high frequency of transfer of NTP16 would suggest a donation mechanism operates (Clarke and Warren, *Ann. Rev. Genet.*, *13*, 99, 1979). However, NTP16DNA is often found to be rearranged in transconjugant cells, and it has been demonstrated that the mobilisation of NTP16 involves its physical association with F, *i.e.*, conduction (Lambert, Hyde and Strike, in press).

Studies so far have shown that the transposable elements $\gamma\delta$ (on F) and IS 176 (on NTP16) are essential for high frequency conduction events. Removal of either transposable element from the system reduces the transfer frequency by a hundred-fold. Evidence indicates that IS 176 is the more active element, having a possible insertional hot-spot within $\gamma\delta$. [The precise interaction between $\gamma\delta$ and IS 176 is still unclear].

Attempts are at present being made to map the cointegrate structures formed as intermediates during transfer. There is some evidence to suggest that NTP16 is often transferred in multimeric form, rather than as a single copy, and resolution of such cointegrates can give rise to more than one form of small plasmid in the recipient cell.

38. Distribution of plasmid protection/mutation genes and their possible assimilation into the bacterial chromosome

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A large number of plasmids carry genes which increase the UV resistance of host strains which harbour them and cause a concommitant increase in induced mutation. Functionally these genetic systems appear to be analogous to the *umuD/C*

operon located at minute 25 on the *E. coli* K12 chromosome. Sequencing of two plasmid systems, the *mucA/B* operon of the Inc N plasmid pKM101 (Perry *et al.*, 1985, *PNAS*, *82*, 4331) and the *impAB* operon of the $IncI_1$ plasmid TP110 reveals large blocks of amino acid homology amongst the products of the *umu*, *muc* and *imp* genes, although DNA homology is minimal. Variability in the regions flanking *umuD/C*, and the fact that these genes are not present in many enterobacteria, including other *E. coli* species, suggests that the *E. coli* K12 chromosomal genes may have arisen by fusion with a replicon carrying these genes.

Amongst the different plasmid groups, specific families of protection mutation genes appear to be present in each incompatibility group. Thus, using *imp* sequences as a probe, homology can be detected in almost all I_1 and B group plasmids so far tested, and in one Inc FIV plasmid, but this probe showed no hybridisation to Inc N plasmids or to any other groups. Similarly, a *muc* probe hybridises specifically to Inc N plasmids. Screening of the Murray collection of strains (isolated in the pre-antibiotic era) reveals the presence of $IncI_1$ and Inc B plasmids carrying *rec/lex* plasmids controlled protection and mutation genes, and the use of *imp* probes to characterise these sequences will be described.

39. Self-incompatibility in *Brassica napus*: A two locus sporophytic system

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Brassica napus is an amphidiploid which is usually self-compatible although its parent species, *B. campestris* and *B. oleracea*, each possess a single locus sporophytic self-incompatibility system. Self-incompatible *B. napus* plants have been found in old cultivars of swedes and oilseed rape, obtained through introgression with *B. campestris* or synthesised by intercrossing the parental species (in some cases using parents of known S-allele status). Analysis of these plants showed that two S-loci could be active in *B. napus* and that both S-specificities were expressed independantly. Evidence was also obtained that alleles at one S-locus may be dominant in either pollen or pistil to those at the other S-locus.

40. Self-incompatibility in *Papaver rhoeas*: I. Characterization of the stigmatic component

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Self-incompatibility in *Papaver rhoeas* is controlled by a single multi-allelic gene (S−) and control of the pollen is gametophytic. We have developed an *in vitro* assay for the stigmatic component of the S-gene. This assay permits the three kinds of reaction characteristic of a gametophytic incompatibility system to be clearly distinguished *in vitro*. Extracts prepared from mature stigma tissue have been fractionated. Use of the assay to monitor S-activity has thereby permitted identification of a glycoprotein which exhibits the specific activity expected of a stigmatic S-gene product.

Details of the assay procedure will be presented together with the results of the tissue fractionation including recent characterisation of the S-glycoprotein from *P. rhoeas*.

41. Self-incompatibility in *Papaver rhoeas*: II. Cloning of stigma specific genes

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We are currently investigating the molecular basis of self-incompatibility in *Papaver rhoeas*. Using the *in vitro* assay described in the accompanying abstract we have established the time of expression of the stigmatic S-gene. In an attempt to clone the stigmatic S-gene and identify other genes expressed at the same stage of stigma development which may play a role in the process of fertilization, we have isolated mRNA from stigmas one day prior to anthesis. A cDNA library of approximately 5×10^6 clones was prepared from this mRNA. A portion of the library was then transferred to duplicate nitrocellulose filters and differentially probed using radioactively labelled cDNA prepared from the same stigma mRNA and from leaf mRNA. Putative stigma specific clones were then rescreened and categorised by cross hybridisation, partial nucleotide sequencing and Southern blotting.

The temporal expression of the mRNA species encoded by the various categories of clones was then investigated by Northern blotting.

A major objective of the cloning was to obtain a cDNA encoding the stigmatic S-gene. In order to determine if the gene has indeed been cloned, the various categories of clone are currently being screened both immunologically and by Southern hybridisation to determine linkage to the chromosomal S-gene.

Details of these experiments and current progress will be presented.

42. Closely related *Brassica* S alleles and the determination of allelic specificity

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A cDNA clone encoding the S_{29} S locus-specific glycoprotein (SLSG) of *Brassica oleracea* var *alboglabra* has been isolated and its nucleotide sequence determined. The predicted protein sequence is, on average, 40 per cent diverged from other *Brassica* SLSGs but shares the emergent features of a signal peptide, an alternating pattern of sequence conservation and divergence, a unique potential N-glycosylation profile and conserved cysteine residues in its C-terminal half. The cDNA probe revealed a single-copy organisation in genomic blots of various *Brassica* S genotypes. This allowed the isolation of a genomic clone of the cross-compatible S_{63} allele. The gene has a contiguous coding region and, remarkably is 99 per cent homologous to the S_{29} sequence. One of only ten amino acid differences between the two eliminates a potential N-glycosylation site in the S_{63} sequence, suggesting that the disposition of sugar residues is a necessary, and perhaps sufficient, determinant of compatibility specificity. This hypothesis can be tested by the transfer of mutagenised S gene constructs into plants and the appropriate test crosses on regenerants.

43. Electrophoretic evidence for the origin of radiate groundsel

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Senecio vulgaris L. (the common groundsel) forms populations which are polymorphic for capitulum type in Britain. The rarer radiate morph is of recent origin and was first reported in 1866. Starch gel electrophoresis has been used to examine the theory that this morph evolved following introgression of *S. squalidus* genes into non-radiate *S. vulgaris* (see Ingram, Weir and Abbott, *New Phytol.*, *84*, 543, 1980). Staining for esterase isozymes using β-napthyl acetate as substrate showed that the radiate and non-radiate morphs of *S. vulgaris* usually possess two β-esterase loci. In contrast *S. squalidus* possesses three such loci, two of which correspond to those in *S. vulgaris*. In one *S. vulgaris* population from York, England, the radiate morph was found to contain all three β-esterase loci present in *S. squalidus*. It is concluded that the York radiate variant contains more *S. squalidus* genes than other forms of the radiate morph and is representative of an early stage in the origin of radiate *S. vulgaris* via introgression of *S. squalidus* into *S. vulgaris*.

44. Interspecific incompatibility between *Brassica napus* and *B. oleracea*

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Brassica napus is an amphidiploid species which may be resynthesized by intercrossing the parental species, *B. campestris* and *B. oleracea*. While the two parental species are self-incompatible, each possessing a single locus sporophytic self-incompatibility system, *B. napus* is usually self-compatible. Test crosses between cultivars of each of these three species showed that pollen from *B. oleracea* usually failed to penetrate the stigmatic surface of *B. napus* pistils although all other combinations of interspecific crosses were compatible with respect to pollen tube penetration. Three out of four synthetic *B. napus* lines, obtained by embryo culture following interspecific pollination of *B. campestris* with *B. oleracea*, were also incompatible when pollinated with *B. oleracea* pollen although the parental lines used were reciprocally cross-compatible. However, the fourth synthetic was reciprocally compatible with *B. oleracea*. The interspecific incompatibility could be overcome by bud pollination or by treatment with cycloheximide as is the case for intra-specific incompatibility in *Brassica* species.

45. The number and frequency of incompatibility alleles in two populations of *Lolium perenne*

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Theoretical considerations lead us to expect that the number of alleles that can be maintained at each locus in a population of a self-incompatible grass species will be slightly greater than half the number that can be maintained in a population of a species with a one-locus system that is of the same size; and that the frequencies of these alleles will be approximately equal at equilibrium. The results obtained from an analysis of the number and frequency of alleles in a very long established natural population of ryegrass at North Meadow suggest that, contrary to expectation, the number of alleles at each locus is approximately 40–45, which is the same as we have found in populations of poppies, which posses a one-locus system; and that the frequencies of the alleles at each locus are significantly unequal.

A similar analysis of a varietal population, "Mascot", suggests that the number of S-alleles it contains is similar to the number of S-alleles in the North Meadow population, though the number of Z-alleles is somewhat less than this. Since this population was founded from just five plants, which between them could not have contained more than 10 alleles at each locus, it is clear that there must have been a considerable immigration of alleles during the course of seed multiplication.

46. Transfer of self-fertility from *Lolium temulentum* to *L. perenne* and *L. multiflorum*

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The important *Lolium* species, *L. perenne* and *L. multiflorum* are outbreeders with a genetically controlled, two locus gametophytic incompatibility system which limits the production of superior recombinant inbred lines. Within the genus a number of inbreeding species occur.

By hybridisation and backcrossing coupled with the use of UV fluorescence tests for pollen/stigma compatibility, the self fertility of *Lolium temulentum* has been shown to be controlled by a single gene and successfully incorporated into the genome of *L. multiflorum* and *L. perenne*. The Sf gene acts gametophytically and it is tentatively suggested to be allelic to or closely linked to the Z locus of the SZ system. It is also linked to the isozyme locus GOT/3. Plants produced were chasmogamous outcrossing readily, but under isolation conditions plants classified as self-compatible by *in vitro* pollination tests set large quantities of self seed (up to 72 per cent). This backcross material provides for transfer of self-fertility into any desired genotype and thus should have important implications for the production of inbred line in the ryegrasses.

47. A transmissible plasmid conferring tetracycline resistance in the obligate anaerobe *Bacteroides ruminicola*

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Obligately anaerobic bacteria of the genus *Bacteroides* are important and abundant inhabitants of the rumen and hind gut of mammals. They are phylogenetically remote from the better studied groups of facultatively anaerobic gut bacteria (*e.g.*, enterobacteria). We have recently demonstrated interstrain transfer of tetracycline resistance and of plasmid DNA in anaerobic matings in the rumen species *B. ruminicola*. The donor strain carried multiple plasmids one of which (a 19·5 kbp plasmid designated pRR14) was shown to be associated with resistance transfer, and to be self-transmissible. Loss of resistance occurred spontaneously at low frequency in transconjugants, in the absence of positive selection, and was accompanied by loss of pRR14. In contrast, in strains of the related colonic species of *Bacteroides* tetracycline resistance is reported to reside mainly on self-transmissible chromosomal elements. The functions of two smaller plasmids present in the *B. ruminicola* donor strain, which can be present in unusually high copy numbers, are not known.

48. Analysis of the structural gene for the calcium-binding protein from *Saccharopolyspora erythraea*

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The gene for a calcium-binding protein from *Saccharopolyspora erythraea* has been cloned in *Escherichia coli* recently and sequenced (Swan *et al.*, *Nature*, *329*, 84–85, 1987). The inferred protein sequence has revealed the presence of four potential "EF" hand calcium-binding loops, a structural motif common to calcium-regulated proteins in eukaryotes but novel in bacteria. We describe here the sequencing and analysis of the DNA regions flanking the structural gene and the cloning of the gene in *Streptomyces lividans*.

Computer-assisted analysis of the DNA sequence indicated that potential hairpin structures are located both 5′, and immediately 3′ to the structural gene. The latter is presumably a terminator of transcription, but the role, if any, of the 5′ inverted repeat is not known, although the run of six T residues which follows it hints at a regulatory role. Mapping of the RNA transcript is in progress. A 3·3 kb fragment of DNA containing the gene has been subcloned and expressed in *Streptomyces lividans* using the vector pIJ702.

49. HLA class II restriction fragment length polymorphisms in rheumatoid arthritis

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There is a well documented association between the HLA (Human Leukocyte Antigen) class II DR4 antigen and susceptibility to rheumatoid arthritis (RA) in most ethnic groups. However, this association is weak (Relative Risk = 4) and HLA-DR4 is known to be heterogeneous at the serological, cellular, biochemical and molecular genetic levels. Also one-third of RA patients do not carry the HLA-DR4 antigen. Therefore stronger HLA and RA associations may be detectable by a direct approach at the gene level. In this study we have utilized HLA class II gene probing technology, Southern blotting and restriction fragment length polymorphism (RFLP) analysis of RA patients and health control individuals in a search for such genetic factors.

Five HLA class II gene probes were used (DRβ, DQα, DQβ, DPα and DPβ) in conjunction with 16 different restriction enzymes. RFLP's were detected with most of these gene probes and particular restriction enzymes. In the majority of such cases there was no significant association with RA. An exception was a 7·2 kb *Dra* IDQβ-associated fragment, which showed a negative association with RA ($p = 0{\cdot}01$–$0{\cdot}05$), while other *Dra* IDQβ-associated fragments showed associations with RA which failed to reach significance. RFLP analysis of 8 HLA-DR4 homozygous typing cell lines showed that none of these RFLP's were associated with the HLA-Dw antigen series and were thus novel genetic markers.

We conclude that HLA class II RA-associated RFLP's are detectable but are scarce and show relatively weak associations with RA. Nevertheless, our data implicate polymorphisms of the HLA-DQβ gene in the aetiology of RA.

50. Sym plasmid variation in *Rhizobium leguminosarum* populations is correlated with chromosomal genotype

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Sym plasmids of *Rhizobium* carry genes essential for symbiosis with legumes. Some well-studied examples are mobilizable or self-transmissible in laboratory crosses. Are all natural Sym plasmids closely similar to the standard laboratory examples, and is plasmid transfer a frequent event in field populations?

To address these questions, the genetic diversity of Sym plasmids was investigated in a total of 85 nodule isolates sampled from two field populations of *R. leguminosarum* biovar *viceae*. These had previously been characterized for electrophoretic alleles at chromosomally-encoded enzyme loci, which showed strong interlocus correlations. Cloned DNA fragments from the *nod* and *nif* gene region of the Sym plasmid pRL1JI were used as

hybridization probes to identify variation in the restriction fragment sizes of the homologous DNA of the isolates.

Many distinct Sym plasmid variants were identified, and their distribution across chromosomal genotypes was far from random. Both field populations (20 km apart) had a similar array of plasmid types and the same correlations between plasmid and chromosome. This suggests that there are barriers to successful transfer or function of Sym plasmids within *R. leguminosarum* populations.

51. Sym plasmid transfer between diverse *Rhizobium leguminosarum* genotypes

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Rhizobium leguminosarum biovar *viceae* isolates have been found to fall into several distinct groups with respect to both their chromosomal and plasmid genotypes. Furthermore, a strong correlation between chromosomal genotype and plasmid group was noted, sugggesting that: (a) natural plasmid transfer is so infrequent as to allow distinct chromosome/plasmid combinations to become established; or (b) plasmid host range is restricted to certain chromosomal genotypes; or (c) Sym genes are adapted to specific chromosomal genotypes so that only certain combinations are successful in nodulation.

To test these hypotheses, *in vitro* conjugative crosses were attempted between representative isolates both as donors (with Tn5 marked Sym plasmids) and as recipients. All strains examined were able to donate their Sym plasmids in certain crosses, but strains differed markedly in their ability to act as donors or recipients. Sym plasmids were transferable to chromosomal backgrounds in which they were not naturally found. This suggests that the observed distribution of Sym plasmids is not due to chromosome-specific barriers to plasmid transfer.

52. Cloning and expression in *E. coli* of an aminoglycoside resistance gene from *Micromonospora*

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A gene (*kgm*) determining resistance to aminoglycoside antibiotics of the kanamycin and gentamycin families has been cloned in *Streptomyces lividans*. This gene originated in *Micromonospora purpurea*, the producer of gentamicin, and encodes a methylase enzyme that acts upon 16S ribosomal RNA, thereby rendering such ribosomes resistant to specific aminoglycosides.

The methylase gene has been inserted into pUC18 and expressed in *E. coli* as a fusion protein. Both strands of the gene have been sequenced in totally overlapping fashion and the sites of initiation and termination of transcription determined by S1 mapping. It is proposed that expression of *kgm* is subject to translational autoregulation.

53. Resistance to novobiocin in *Streptomyces sphaeroides*, producer of novobiocin

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Shotgun cloning of DNA fragments from *Streptomyces sphaeroides* in *Streptomyces lividans* generated novobiocin-resistant strains, in which DNA gyrase activity was resistant to the drug. Southern analysis revealed that the cloned DNA included a *gyrB* gene encoding the B subunit of DNA gyrase from *S. sphaeroides*. Properties of the purified enzyme will be presented together with aspects of genomic organisation in *S. sphaeroides*.

54. The regulation of a thiostrepton induced promoter in *Streptomyces lividans*

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Since the primary lethal activity of many antibiotics is to inhibit translation, other interesting and useful effects may have been overlooked. These secondary effects might be observed if the ribosome is modified by a drug resistance gene to prevent interaction of the antibiotic with the target.

Ribosomes found in streptomycetes containing the thiostrepton resistance gene (*tsr*) are methylated at a specific pentose residue of the 23S rRNA so as to prevent binding of the antibiotic. In addition to inhibiting translocation and acyl-tRNA charging on the ribosome, thiostrepton inhibits (p)ppGpp synthase and thus interferes with the stringent response. In the course of experiments designed to express foreign genes in *S. lividans*, we noticed that thiostrepton also induced the synthesis of at least two proteins of molecular weights *ca.* 17 kdal and 19 kdal. Coomassie blue staining of SDS-PAGE gels showed that while the 19 kdal protein could not be detected in mycelia which had been grown in antibiotic-free medium, in mycelia which had been grown in the presence of thiostrepton the 19 kdal protein was a major band (*ca.* 10 per cent of total cell protein). The 17 kdal protein was not as abundantly induced. In order to identify the gene which coded for the 19 kdal band, we purified the protein, determined its N-terminal sequence, and then used this sequence to design an oligonucleotide probe. The probe was used to isolate hybridization positive clones from a bank of *S. lividans* genomic DNA cloned in the bacteriophage lambda vector, EMBL4. Subcloning in pUC19 followed by nucleotide sequence analysis confirmed that we had indeed cloned the gene corresponding to the protein induced by thiostrepton and that it did not correspond to the product of the *tsr* gene. Northern blots of RNA extracted from *S. lividans*/pIJ702 showed that a *ca.* 1 kb mRNA which hybridized to this probe was induced more than 1000 fold by thiostrepton. We think that the thiostrepton induced promoter will be a very useful tool for studies of endogeneous and foreign gene expression in streptomycetes.

55. Characterisation of the *Streptomyces coelicolor* A3(2) minicircle

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The minicircle is a transposable element present in two distinct copies (A and B) on the *Streptomyces coelicolor* A3(2) chromosome; a circular form with very low copy number is also detectable (Lydiate *et al.*, *Mol. Gen. Genet.*, *203*, 879–881, 1986). It encodes a function allowing integration into the chromosome of a number of *Streptomyces* species. In *S. lividans* the integration most frequently occurs into a preferred site.

The nucleotide sequence of a cloned copy of the minicircle has been determined and three probable coding open reading frames identified. The sequence of the DNA flanking the *S. coelicolor* linear copy A and the sequence of the *S. lividans* preferred site, both before and after minicircle integration, has also been determined. There are no perfect inverted repeats at the ends of the element and there is no extensive homology between the minicircle and its preferred integration site. The minicircle has been found to be related to other *Streptomyces* transposable elements. Disruption of the minicircle sequence at various restriction sites has implicated the largest of the open reading frames in the integration function. Plasmid constructs containing the minicircle may be useful as integrative vectors in a wide range of *Streptomyces* species.

56. The gene for orsellinic acid synthase from *S. curacoi*

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Many secondary metabolites, among them a lot of antibiotics, are synthesised by the polyketide pathway. Some genes for polyketide synthases have been cloned from *Streptomyces*. To investigate the mechanism of polyketide biosynthesis, we decided to clone the gene for orsellinic acid synthase from *S. curacoi*. Orsellinic acid, which is the simplest possible aromatic polyketide, is the precursor of the aglycone moiety of curamycin.

As Southern blots showed that an about 3 kb long PstI fragment was homologous to the *Act*I probe, this fragment was chosen for further studies. A restriction map and sequence of this fragment is presented. Subcloning and hybridisation showed that an internal fragment was responsible for the *act*I homology. The sequence is compared to other known polyketide synthase sequences.

57. Analysis of *tuf*-genes from *Streptomyces ramocissimus*

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Streptomyces ramocissimus produces the antibiotic kirromycin, which acts on the elongation factor EF-Tu. The sensitivity to kirromycin of EF-Tu from different bacteria has been shown to vary. Surprisingly, EF-Tu isolated from *S. ramocissimus* is sensitive to kirromycin *in vitro*. Attempts to isolate kirromycin-resistant mutants of *S. ramocissimus* have failed so far. In general, a *tuf*-gene coding for a kirromycin-sensitive RF-Tu protein is dominant over a *tuf*-gene coding for a resistant protein. *E. coli* harbours two *tuf*-genes, which have to be altered both in order to obtain phenotypic expression of kirromycin-resistance.

Using *tuf*-DNA from *E. coli* as a heterologous probe on Southern blots of *S. ramocissimus* genomic DNA we obtained multiple hybridization signals. Two restriction fragments were cloned and each proved to contain a complete *tuf*-gene. This finding, together with the data from Southern hybridizations, leads us to presume the existence of a third *tuf*-gene on the genome of *S. ramocissimus*. DNA sequencing of the two cloned genes shows that they code for proteins with a rather dissimilar primary structure. In contrast, the proteins encoded by both *E. coli tuf*-genes differ at the C-terminal residue only.

58. Molecular relationship of bacteriophages of the actinomycete genus *Faenia*

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20 actinophages of the thermophilic *Faenia rectivirgula* (some also infecting *Saccharopolyspora erythraea*, formerly *Streptomyces erythraeus*, or *Saccharopolyspora hirsuta*) from four groups with regard to their DNA-homology to ϕFR114, the best characterized phage among them:

(1) The phages (including ϕFR114) exhibit a strong homology to ϕFR114. In some cases, however, the "central region" (CR) shows less homology: three different CRs are distinguished this way. The phages are *temperate* and cannot infect strains with a ϕFR114 prophage.
(2) The phages contain only small regions with a *pronounced* homology to ϕFR114. They are *temperate* and cannot infect strains with a ϕFR114 prophage.
(3) These phages contain only small regions with a *weak* homology to ϕFR114. Although they are *virulent* they cannot lyse strains with a ϕFR114 prophage.
(4) The three *virulent* phages are closely related to each other and are not repressed by a ϕFR114 prophage. Two of them show a strong homology to the CR of ϕFR114, the third none at all.

Five regions of the ϕFR114-genome are found on different phages of group 2, 3 or 4, one being common to all phages repressed by a ϕFR114 prophage. These and other possible modules are discussed with respect to phage evolution (Schneider *et al.*, this meeting).

59. Protoplasts of *Faenia rectivirgula*—formation, regeneration, and transformation

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A host-vector system is needed for the genetic study of a group of closely related actinophages (Schneider and Kutzner, this meeting). *Faenia rectivirgula* is a suitable host for all 20 phages under investigation, although some of them were isolated for *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*). *Saccharopolyspora hirsuta*, another host for some of the phages, has a very strong restriction barrier. Therefore, *F. rectivirgula* DSM 43747 was selected as the host-strain for host-vector experiments which are discussed in this paper:

(1) Formation and regeneration of protoplasts
(2) Transformation of protoplasts with the *Streptomyces* plasmid vectors pIJ702 and pWOR120
(3) Instability of pIJ702 and pWOR120 in *Faenia rectivirgula*
(4) Attempts to construct a vector based on the *Faenia* plasmid pFR1

It could be demonstrated that the thermophilic *F. rectivirgula* can be used as a host for transformation experiments. Using the vectors available so far, however, transformation is less efficient than in well established systems as *Streptomyces lividans*. A similarly low efficiency of transformation was reported for other actinomycetes such as *Thermomonospora*. The possible development of a plasmid vector based on the 4 kb plasmid pFR1 from *Faenia rectivirgula* is discussed with regard to these problems.

60. Characterization of four new plasmids of *Streptomyces coelicolor* (Müller)

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This communication reports on the comparison of several plasmids from *Streptomyces coelicolor* (Müller), a species which is different from *S. coelicolor* A3(2). 8 out of 54 strains were shown to contain one or more plasmids (Korn-Wendisch and Kutzner, this meeting) which are compared by restriction analysis, and in come cases by restriction mapping.

The two strains DSM 40130 and DSM 40131 both carry the plasmid pSC130 (35 kb); the restriction map of this plasmid is presented. Strain DSM 40675 harbours three plasmids: the two smaller ones (pSC675-3, 6·4 kb; pSC675-2, 9·2 kb) occur irregularly in strains derived from single spores or regenerated protoplasts, whereas the larger pSC675-1 (25·5 kb) proved to be stable. Comparison of the restriction maps suggested a possible relationship between pSC675-1 and pSC675-2. However, homology studies using cloned fragments of pSC675-1 and pSC675-3 as biotinylated probes demonstrated that the three plasmids are not related.

In addition to the molecular studies presented above the different plasmids were used in mating experiments: no pock formation was detected with *S. lividans* or *S. coelicolor* (Müller) as recipient strains.

61. Transfection of *Faenia rectivirgula* with phage DNA from different actinomycete genera

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The investigation of 20 actinophages of the thermophilic *Faenia rectivirgula* and the related species *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*) and *Saccharospolyspora hirsuta* had revealed several possible modules which are distributed among the phages. One module seems to be correlated to prophage regulation as indicated by a prophage mediated immunity against all phages with the respective module (Schneider and Kutzner, this meeting).

It was postulated that the exchange of functional modules was (or still is) involved in the evolution of the phages under investigation, similar to the situation known from lambdoid phages. If this hypothesis is correct, related modules might be distributed within other bacteriophages of actinomycete genera which are related to *Faenia* but not within the natural host range of the *Faenia*-phages.

In this paper we report on the transfection of *Faenia*-proptoplasts (Gayer-Herkert *et al.*, this meeting) with the DNA from phages of *Saccharomonospora*, *Thermomonospora* and *Streptomyces coelicolor* (Müller). Furthermore, phage host ranges and homology-studies for the detection of possible relationships between all these phages are discussed.

62. The metabolism of tyrosine in *Streptomyces michiganensis*

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Tyrosine is used by *Sm. michiganensis* for two purposes: (1) The amino acid can serve as the only C- and N-source for growth in synthetic media; (2) alternatively, tyrosine is used for the formation of melanin. To investigate the regulation of tyrosine metabolism—*i.e.*, (i) degradation and use as growth substrate and/or (ii) oxidation and polymerisation to melanin—we first studied the degradation pathway of tyrosine. Enzymatic and polarographic tests (Clark oxygen electrode) with tyrosine-adapted cells suggest the following metabolic sequence: Tyrosine → p-hydroxyphenylpyruvate → homogentisate → ringcleavage.

The homogentisate oxidizing activity of crude extracts was lost after dialysis and could be restored by addition of Fe^{2+}; this could not be replaced by Fe^{3+}. Since tyrosine is a poor substrate for growth, (possibly because of a low uptake-rate), a suitable method had to be developed for the isolation of mutants which are defective in tyrosine degradation (td). These mutants were tested for accumulation of intermediates and complementation.

Melanin formation: Tyrosinase, the enzyme responsible for the formation of melanin is a

Cu^{2+}-containing monophenol-monooxigenase (E.C. 1.14.18.1). In *Sm. michiganensis* this enzyme is produced constitutively, *i.e.*, in a synthetic medium with glycerol and nitrate as C- and N-source in contrast to *Sm. glaucescens*, where the enzyme is induced by various amino acids. However in *Sm. michiganensis* the tyrosinase underlies a catabolite repression by ammonia. For further investigation mel-negative mutants were isolated. Although such mutants arise spontaneously at a relative high rate (0·1 per cent), UV- and NTG-treatment were used to increase their number.

63. Distribution and stability of plasmids in *Streptomyces*

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Altogether about 250 strains of several species of *Streptomyces* have been screened for the occurrence of circular plasmids using alkaline lysis and agarose gel electophoresis. The various species differed markedly in harbouring plasmids, *e.g.*, more that 30 per cent of the 27 strains of *Sm. violaceoruber* and of 17 streptomycetes with green aerial mycelium contain one or more plasmids, whereas none could be detected in any of 31 strains of *Sm. albus*.

The stability of plasmids was studied in subcultures conserved as lyophils, soil cultures, dried on beads, in glycerol or above liquid nitrogen: large plasmids turned out to be stable while some variation was observed in strains with several smaller plasmids. Finally, the influence of the regeneration of protoplasts was examined: again, large plasmids proved to be stably maintained whereas smaller plasmids frequently disappeared, *e.g.*, in *Sm. lividans* (DSM 40434) and *Sm. coelicolor* Müller (DSM 40675) 80 per cent and 90 per cent respectively (Held *et al.*, this meeting).

64. Overproduction of *Streptomyces* extracellular enzymes cloned into multicopy plasmids: influence of the culture conditions and of the host organism

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Genetically engineered *Streptomyces lividans* strains overproducing extracellular enzymes (β-lactamase or DD-carboxypeptidase) exhibited time-course productions which culminate after 48 to 72 h, at a maximum of 40 mg of enzyme/litre of culture. Later, the enzyme content of the culture drastically dropped (Dehottay *et al.*, *Gene.*, *42*, 31-36, 1986). This sudden decrease could only partly be explained by the evolution of the *p*H of the medium, this *p*H effect being reinforced by the action of an inhibitor of the serine active enzymes, synthesized by *S. lividans*.

By modifying the conditions of culture (buffer, carbon or nitrogen sources), the loss of enzyme activity was reduced and the maximum of enzyme production was increased up to 160 mg/litre.

In the case of the plasmid pDML6 carrying the *Streptomyces albus* G β-lactamase gene, introduction into *S. albus* G itself results in an enzymic yield of more than 1 g/litre.

The cultivation of our overproducing strains as immobilized cells, in a semi-continuous way, allowed a reduction of the peptone content of the medium, without any decrease of the enzymic yield. Such a system has been kept up for 40 days, a slowing down of the production being observed from the 25th day only.

65. The β-lactamase gene of *Streptomyces cacaoi* nucleotide sequence and transcriptional analysis

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The gene coding for the extracellular β-lactamase of *Streptomyces cacaoi* has been previously cloned into *Streptomyces lividans*. It was shown that the gene was expressed and its product excreted. Different subclones, all of them borne by pIJ702 derivatives, gave different levels of expression

(Lenzini *et al.*, *J. Gen. Microbiol.*, *133*, 2915–2920, 1987).

Experiments of S1 mapping have shown that three of these subclones, pDML51, pDML52 and pMCP38 shared the same transcription startpoint situated 224/225 bp upstream from the codon corresponding to the N-terminus of the mature protein. A promoter sequence was identified and the translation startpoint for the precursor protein, defining the signal sequence, will be discussed.

On the other hand, a fourth construct pDML39 started to transcribe the gene upstream to the startpoint mentioned here above. The use of a terminator of transcription suggested that, in this last subclone, the *S. cacaoi* gene was controlled by a promoter belonging to the vector.

66. Expression into *Streptomyces lividans* of foreign genes coding for extracellular β-lactamases, use of *Streptomyces* secretion vectors

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The genes *amp*C from *Enterobacter cloacae* P99, and *bla*P from *Bacillus licheniformis* 749/C which code for extracellular β-lactamase have been introduced into *Streptomyces lividans*, by using *Streptomyces* plasmid vectors pIJ702 and pIJ385, respectively. The genes were supposedly under the control of non-*Streptomyces* promoters and led by their own signal sequences. In both cases, less than 1 mg of enzyme was produced by litre of culture and about the half of the activity remained cell-associated.

Two *Streptomyces* secretion vectors have been constructed by using the promoter regions and the signal sequences from the gene coding for the *Streptomyces albus* G β-lactamase (Dehottay *et al.*, *Eur. J. Biochem.*, *166*, 345–350, 1987) giving the vector pDML9 and from the gene coding for the *Streptomyces R61 DD-carboxypeptidase* (*Duez et al.*, *Eur. J. Biochem.*, *162*, 509–518, 1987) giving the vector pDML116. The foreign genes tested were the *bla*P gene from *Bacillus licheniformis* and the Ap^R gene from pBR322. The production of foreign extracellular proteins *via* these secretion vectors appeared to be lower than the production of *Streptomyces* proteins.

67. Cloning and expression of the β-lactamase gene of *Actinomadura* R39 in *Streptomyces lividans*

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The *Streptomyces* promoter probe plasmid pIJ424 has been used to show that the promoters from the *Actinomadura* R39 genome were efficient in a *Streptomyces lividans* cell. The so-constituted libraries were then screened by the nitrocefin test and several β-lactamase excreting *S. lividans* colonies were detected.

The best β-lactamase producer out of these clones, *S. lividans* CM3, excreted 100 times more β-lactamase than the original *Actinomadura* R39 strain. The enzyme synthesized by *S. lividans* CM3 has been thoroughly characterized and was shown to be identical to the original R39 protein, in all of the assays performed.

The plasmid pDML150 extracted from *S. lividans* CM3 carried a 1·8 kb insert which was further subcloned into *Escherichia coli*, *via* pBR322. No expression of the gene occured in *E. coli*. The restriction map of this insert has been established and preliminary data about the nucleotide sequence will be presented. They revealed a striking similarity with the *Bacillus licheniformis* β-lactamase.

68. New data on the formation and mode of action of factor C

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Introduction of two new experimental techniques widened the possibilities to investigate the effect and formation of the endogenous regulatory protein factor C in *Streptomyces griseus* (Szabó *et al.*, *Acta Biol. Acad. Sci. Hung.*, *18*, 237–243, 1967 and Bíró *et al.*, *Eur, J. Biochem.*, *103*, 359–363, 1980).

Rapid changes in the potassium equilibrium between *Streptomyces* mycelium and its medium can be measured by means of ion-selective membrane electrode. Factor C causes a release of intracellular K^+ within a few minutes after its addition to mycelial suspensions in concentration

as low as 0·7 ng/ml. In *Amy-Streptomyces* strains the effect of factor C and factor A was found antagonistic.

Monoclonal antibody was developed. ELISA assay was applied. Factor C antigen was detected also in the fermentation liquids of *S. griseus* No. 52-1, the indicator strain which otherwise never produced factor C in a cytomorphologically active form. After the addition of factor C, the indicator strain also started to produce actively the cytomorphologically active substance and the ratio of the cytomorphological activity to factor C antigen changed by a characteristic way.

69. Sequence of a *Streptomyces griseus* streptomycin phosphotransferase: Sequence homologies between aminoglycoside phosphotransferases and vertebrate protein kinases

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We have previously cloned a streptomycin phosphotransferase gene from *Streptomyces griseus*. The sequence has now been determined and confirms that of Distler *et al.* (*Molec. Gen. Genet., 208*, 204–210, 1987). A computer-assisted comparison was made of this sequence with other aminoglycoside (streptomycin, neomycin/kanamycin, hygromycin B and viomycin) phosphotransferase sequences from both Gram-positive and Gram-negative bacteria. For the streptomycin, neomycin/kanamycin and Gram-positive hygromycin B phosphotransferases, a basic structure A-B-C-D-E was revealed, where B and D were conserved between all these sequences, while A, C and E divided between the streptomycin and hygromycin B phosphotransferases on the one hand and the neomycin/kanamycin ones on the other. The former blocks may therefore represent domains concerned with ATP binding and phosphate transfer; the latter, domains involved in antibiotic recognition.

A composite database was then searched for sequences similar to six 12-residue consensus matrices constructed from portions of blocks B and D within the aminoglycoside phosphotransferase sequences. The hit lists revealed that the only protein types appearing consistently among the highest-scoring hits were vertebrate protein kinases (of both the serine/threonine- and tyrosine-specific types). Similar but less extensive sequence similarities were also reported by Distler *et al.* (op. cit.) and have also been noted by Brenner (*Nature, 329*, 21, 1987). Possible implications of these results regarding the evolution of these sequences will be discussed.

70. "Strong incompatibility" between derivatives of the *Streptomyces* multi-copy plasmid pIJ101

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Some derivatives of pIJ101, a 8·9 kb *Streptomyces* multi-copy plasmid, can co-exist with each other at similar copy numbers but others are strongly incompatible. The DNA sequence, *sti*, which causes this "strong incompatibility" was localized on a DNA segment of about 200 bp which lies in a region outside the basic replicon of pIJ101. The *sti* function is active only when the DNA fragment carrying it is present in the natural orientation with respect to the basic replicon region of pIJ101. Plasmids which either both possess *sti* in the correct orientation (Sti+) or both lack *sti* (Sti−) can co-exist, but Sti+ and Sti− plasmids cannot; in this case the Sti+ plasmid is retained and the Sti− plasmid is rapidly lost. Plasmids with *sti* in inverted orientation are also Sti−. We have called this phenomenon "strong incompatibility" to distinguish it from classical incompatibility where identical or related plasmids are incompatible and dissimilar plasmids are compatible. It is likely that pIJ101 replicates *via* a single-stranded intermediate (rolling circle replication) and that *sti* is a site where the synthesis of the second (lagging) DNA strand is initiated, because plasmids which lack *sti* in the correct orientation accumulate more single-stranded plasmid DNA than Sti+ plasmids. The copy number of pIJ101 and its derivatives is influenced by *sti* and by an additional *trans*-acting function (*cop*).

71. Co-integrate formation between *Streptomyces* plasmids stimulated by a sequence of the multi-copy plasmid pIJ101

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A DNA fragment of the *Streptomyces* multi-copy plasmid pIJ101 promotes the formation of co-integrates between pairs of plasmids which contain this fragment in opposite orientations but not between pairs of plasmids with the fragment in the same orientation relative to the essential replication functions of pIJ101. The observed co-integrate plasmids probably accumulate in the cells because they have an advantage over the parental plasmids.

72. pIJ699, a multi-copy positive selection plasmid vector for *Streptomyces*

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pIJ699, a plasmid vector which gives positive selection for cloned DNA, was constructed using the replication functions of the *Streptomyces* wide host range multi-copy plasmid pIJ101. The selection for inserts is based on the principle that plasmids with long uninterrupted perfect palindromes (inverted repeats) are not "viable" in bacteria (Collins *et al.*, *Gene*, *19*, 139–146, 1982; Hagan and Warren, *Gene*, *19*, 147–151, 1982). For cloning, pIJ699 is digested with *Bgl*II. This produces two fragments, one of which is the vector and the other is a "stuffer" which is needed to keep the inverted repeat sequences apart. The vector fragment is separated from the "stuffer" fragment and ligated with the DNA to be cloned. Plasmids with a fragment of cloned DNA, but not circularized vector fragments, give rise to $Thio^R$ transformants in *Streptomyces lividans*. The inverted repeat sequences contain a strong transcription terminator which reduces transcriptional read-through both in and out of the cloned fragment. This improves the stability of many hybrid plasmids and facilitates the study of the regulation of cloned genes.

73. Site-specific degradation of *Streptomyces lividans* DNA during electrophoresis

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Streptomyces lividans DNA contains a DNA modification which makes it susceptible to double-strand cleavage during electrophoresis in buffers contaminated with ferrous iron (which may be present in some batches of EDTA). The cleavage of the DNA is site-specific and the average fragment size resulting from limit digestion of total *S. lividans* DNA is about 6 kb. DNA from several other *Streptomyces* strains and from *E. coli* is not cleaved under the same conditions. A *S. lividans* mutant has been isolated which lacks the DNA modification.

We suspect that many reports of "poor" preparations of *S. lividans* plasmids may be due to the above effect.

74. Construction of a streptomycin/spectinomycin resistance fragment which is flanked by SP6 and T7 promoter sequences

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The Omega fragment of Prentki and Krisch (*Gene*, *19*, 303–313, 1984) containing a gene conferring streptomycin and spectinomycin resistance flanked by transcriptional terminators has been modified by adding SP6 and T7 promoters and symmetrical polylinkers to its ends. This modified Omega fragment, called Omega SPT, has been specifically designed for inserting into the *Streptomyces* unit-copy plasmid SCP2* and its derivatives to cause mutations which can easily be located in these large replicons. The fragment can be excised from its carrier by digestion with *Bam*HI, *Sma*I or *Sst*I.

The *Sma*I fragment is the most versatile because it can readily be ligated to any blunt-ended DNA fragment. Recombinant plasmids containing Omega SPT can be selected in *E. coli* or *S. lividans* because the spectinomycin/streptomycin resistance gene is expressed in both hosts. Digestion with *Xho*I removes the Omega SPT fragment leaving behind only 24 nucleotides. This facilitates the determination of the site of insertion and may in many cases restore the original phenotype. The SP6 and T7 promoters, which read away from the fragment, can be used as start points for DNA sequencing of the site of insertion and *in vitro* transcription/translation may produce enough protein for the preparation of specific antibodies.

75. *In vivo* genetic recombination in fast growing mycobacteria

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An effective method was developed for spheroplast preparation from fast growing mycobacteria. In our study *Mycobacterium fortuitum*, *Mycobacterium phlei* and unidentified Mycobacterium strains isolated from soil were used. Auxotrophic and antibiotic resistance mutants of the different Mycobacterium strains (Ade^-; Val^-; Pro^-; $Val^- Pro^-$; $Val^- Leu^-$; Str^R) were obtained by chemical mutagenesis of the wild type strains. Spheroplasts were prepared by lysozyme treatment of cells after cultivating in glycine- and vancomycin-containing media.

Spheroplasts of the mutants were mixed in 1 : 1 ratio and were treated with 50 per cent (w/v) polyethylene glycol 6000 for 5 min at 37°C. The recombinants were selected on the basis of genetic markers. The recombination frequency was $5{\cdot}6 \times 10^{-2} - 1{\cdot}6 \times 10^{-3}$. *Mycobacterium* species are widely applied in microbial transformation of steroids. According to our experiments *in vivo* genetic recombination is a useful method to improve industrially-important Mycobacterium strains.

76. Cloning and analysis of an α-amylase gene from *Streptomyces limosus*

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An α-amylase gene (*aml*) of *Streptomyces limosus* ATCC 19778 was cloned in *Streptomyces lividans* 66. Low resolution S1 mapping experiments identified an *aml* transcript 1·8 kb in length and the extracellular enzyme was estimated to be 59 kd in size, suggesting that *aml* was transcribed as a monocistronic mRNA species. Expression of the gene was induced by maltose (or maltodextrins) in *S. limosus* and when *aml* was cloned in *S. lividans* or *Streptomyces coelicolor* A3(2). In *S. limosus* mannitol repressed *aml* expression while glucose had little or no effect; in *S. lividans* and *S. coelicolor* the relative effects of the two sugars were reversed. High resolution S1 mapping experiments indicated that both induction and carbon source repression occurred at the level of transcriptional initiation from a unique promoter of typical prokaryotic appearance. An open reading frame that precedes *aml* shows significant amino acid sequence similarity to both the *gal* and *lac* repressors of *Escherichia coli* and may encode the repressor of the α-amylase gene. Glucose repression in *S. coelicolor* depended on a functional glucose kinase gene. The predicted amino acid sequence of *aml* shows considerable identity to mammalian and invertebrate α-amylases, but not to those from plants, fungi or other non-streptomycete bacteria. Consistent with this is the susceptibility of the enzyme to an inhibitor of mammalian α-amylases.

77. The repressor gene of the *Streptomyces* temperature phage ϕc31

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The nucleotide sequence of the 3·4 kb *Sph*I-G fragment that contains the repressor gene (*c*) of the temperate *Streptomyces* phage ϕc31 was determined. Analysis of this sequence revealed a long open reading frame with protein coding character; sequence changes in *c* point and deletion mutants identified this as the coding region of the repressor. Two of the mutants studied had undergone deletions of 1·1 and 1·4 kb that had occurred across

short direct repeats of 6 bp and 11 bp respectively. Coupled *in vitro* transcription-translation experiments using the cloned *Sph*I-G fragment and *Streptomyces lividans* cell free extracts identified a protein product of approximately 72 kd, in close agreement with that predicted from the nucleotide sequence. A strongly predicted helix-turn-helix motif that may be involved in DNA binding occurred towards the carboxy-terminus of the amino acid sequence. S1 mapping and *in vitro* transcription studied suggest that a complex regulatory region preceeds the coding sequence of the repressor gene. Initial attempts to clone the *Sph*I-G fragment in *Streptomyces* failed; using information gained from the sequence analysis a smaller segment of this DNA fragment was cloned in *S. lividans* and conferred immunity to a clear plaque mutant (*c*1) of ϕc31.

78. Analysis of a phosphinothricin N-acetyltransferase gene from *Streptomyces viridochromogenes* and its use for the construction of herbicide resistant plants

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A 0·8 kb *Bgl*II fragment conferring resistance against phosphinothricin-tripeptide (Ptt) was isolated from *Streptomyces viridochromogenes* Tü494. The fragment contains a gene that codes for a phosphinothricin *N*-acetyltransferase (Strauch *et al.*, *Gene*, in press). The nucleotide sequence of a 1·3 kb *Bgl*II-*Sst*II fragment including the resistance gene was determined according to the method of Maxam and Gilbert. An open reading frame coding for the acetyltransferase was found. The base composition of the gene is typical for *Streptomyces*. The 5′-end of the gene was difficult to determine since two possible translational start codons were found. By defined frame shift mutations a GTG codon was identified as translational start codon. The last codon (ATC) is part of the *Bgl*II site (A′G*ATCT*) at the end of the fragment. A putative ribosome binding site and a sequence which may function as promoter were identified by the comparison with the 3′ terminal sequence of 16S rRNA of *S. lividans* and with the consensus promoter sequence (TTGaca-18 bp-tAGgat) of *Streptomyces* respectively. The amino acid sequence of the phosphinothricin *N*-acetyltransferase derived from the nucleotide sequence consists of 183 amino acids. The molecular mass of the protein is 20·5 kDa. A similar gene was isolated from *S. hygroscopicus* (Thompson *et al.*, *EMBO J. 6,* 2519-2523, 1987). The gene product shows 85 per cent homology on the amino acid level to the *S. viridochromogenes* resistance gene.

As Ptt and phosphinothricin can be used as a non-selective herbicide the acetyltransferase gene was transferred to *Nicotiana tabacum* to construct resistant plants. Using oligonucleotide synthesis the start codon GTG was converted into ATG and fused to the CaMV35 S promoter. Using the binary vector system for *Agrobacterium tumefaciens* the resistance gene was introduced into tobacco cells by leaf disc transformation. The transformed plants were resistant against the herbicide phosphinothricin.

79. Isolation of a phosphinothricin–tripeptide resistance gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Streptomyces lividans* and *Escherichia coli*

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Phosphinothricyl-alanyl-alanine (Phosphinothricin-tripeptide, Ptt) is an antibiotic produced by *S. viridochromogenes* Tü494. The phosphinothricin moiety of Ptt is known for its herbicidal activity. Surprisingly, *S. viridochomogenes* is sensitive against its own antibiotic. Germination of spores is inhibited by low concentrations of Ptt (5 μg/ml). Also mycelial growth of producing cultures is inhibited when plated on minimal medium supplemented with Ptt. We isolated resistant mutants of *S. viridochromogenes* which arose spontaneously with a frequency of about 10^{-4} on plates containing Ptt. Most of these clones are resistant to concentrations of Ptt higher than 100 μg/ml.

Shot-gun cloning experiments with total DNA of one of these resistant clones (ES1) were performed using pEB2 as vector plasmid (Wohlleben *et*

al., 1986, in *Sixth Int. Symp. on Actinomycetes Biology*, pp. 99–101) and *S. lividans* TK23 as recipient. Four out of 20,000 transformants were found to be resistant against Ptt. The Ptt resistant transformants contained the same 4·0 kb insert in the single *Bam*HI site of the cloning vector pEB2. The coding region of the resistance gene could be localized on a 0·8 kb *Bgl*II subfragment. By cloning this fragment into the promoter-probe vector pIJ425 (Ward *et al.*, *Mol. Gen. Genet.*, *203*, 468–478, 1986) it could be demonstrated that the gene is expressed from its own promoter. Subcloning of the *Bgl*II fragment downstream from the *lacZ* promoter in the *E. coli* vector pSVB20 resulted in Ptt resistant *E. coli* clones. The resistance gene codes for a phosphinothricin N-acetyltransferase. This was shown by the formation of N-acetyl-phosphinothricin in crude extracts of Ptt resistant *Streptomyces* and *E. coli* clones.

80. Cloning of a DNA frament of *Streptomyces coelicolor* DSM3030 encoding the production of a blue pigment

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Streptomyces coelicolor "Müller" DSM3030 produces indochrome, a water soluble blue pigment. Indochrome or "amylocyanin" is a mixture of ribosyl-isomere diazaphenol-indophenols (Habermehl *et al.*, *Z. Naturforsch.*, *32b*, 1195–1203, 1977). A genomic library of *S. coelicolor* was established in *S. lividans* using pSG5 based cloning vectors (Muth *et al.*, *Mol. Gen. Genet.*, in press). Screening this gene bank we isolated several dark blue pigmented colonies. A 5·5 kb *Bam*HI fragment was found to be responsible for this phenotype. This DNA fragment was also expressed in many other *Streptomyces* species. Analysis of the pigment production showed that the blue pigment encoded by the recombinant plasmid (pGM98) was not identical with indochrome. Complementation experiments using *S. coelicolor* mutants blocked in indochrome production suggest the cloning of genes involved in indochrome biosynthesis. We therefore conclude that pGM98 mediate the production of a indochrome "precursor" or a modified indochrome molecule.

81. Development of useful cloning vectors based on the *Streptomyces venezuelae* plasmid pSVH1

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Streptomyces venezuelae DSM40755 harbours the endogenous plasmid pSVH1. This plasmid with a size of 12·6 kb has an apparent copy number of 80–100 and can be easily isolated using *E. coli* and *Streptomyces* standard procedures. pSVH1 has a broad host range and is a self-transmissible pock forming plasmid in *S. lividans*.

The replication and transfer regions were characterized by subcloning experiments. The regions required for replication and stable maintenance were determined in a 2·5 kb fragment. The region of transfer was localized on a fragment of 1–1·5 kb.

On the basis of this minimal replicon, *E. coli–Streptomyces*-shuttle vectors as well as vectors for use in *Streptomyces* were constructed. These vectors possess the resistance genes for thiostrepton (*tsr*), neomycin (*aph*I) (Thompson and Gray, *Proc. Natl. Acad. Sci.*, *80*, 5190–5194, 1983) and kanamycin from Tn5 (*aph*II). For cloning experiments various single restriction endonuclease cleavage sites can be used.

Temperature sensitive replication mutants useful for mutational cloning were generated by *in vitro* hydroxylamin mutagenesis. These plasmid mutants were stably maintained at temperatures up to 35°C and lost at temperatures above 37°C.

pSVH1-vectors were successfully used in cloning experiments.

82. Isolation and characterization of a pock-forming plasmid, pBLE1, from a *Streptomycete* soil-isolate

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A new conjugative plasmid, pBLE1, was isolated from a Streptomycete soil-isolate. This plasmid was found to be 8·4 kilobases in size and can be maintained in *Streptomyces lividans* TK24. In mating experiments pBLEI was shown to cause a lethal zygosis phenotype. The plasmid has been restric-

tion-mapped and it has a least four unique cleavage sites. The plasmid copy number is 30–50. The characteristics of pBLE1 may allow its development as a cloning vector useful in the genetic engineering of Streptomycetes.

Various plasmid functions were localized by the mutational cloning of a positive insertion vector pIJ4624 into different sites on pBLE1. pIJ4624 replicates in *E. coli* only, but it also contains some Streptomycete selection markers (Kieser, T., personal communication). The composite plasmids were obtained from *E. coli* and then introduced into *S. lividans* to analyse the effect of the pIJ4624 on pBLE1 functions.

83. Mobilisation of plasmid NTP16 by a variety of conjugative plasmids

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Mobilisation is the process whereby small non-conjugative plasmids can be transferred between bacteria, and is dependent on the activity of a co-resident conjugative plasmid. It is reasonable to assume that the *mob/nic* systems of such plasmids would evolve to be compatible with several different conjugation systems, in order to be transferred to a range of different hosts and environments.

Plasmid NTP16 is a non-conjugative plasmid of 8·8 kb, carrying resistance to ampicillin and kanamycin. It has been found to be efficiently mobilised by conjugative plasmids from a wide variety of Incompatibility groups, (27 plasmids from 20 different Incompatibility groups were investigated). This result contrasts with smaller studies done on other non-conjugative plasmids, which suggest greater specificity.

The high mobilisation frequencies achieved imply that the transfer mechanism is through the *mob/nic* system of NTP16, although mobilisation can also be mediated through the formation of cointegrates between two plasmids.

The *mob/nic* system of NTP16 has been cloned, and is currently being sequenced.

84. Isolation and characterisation of *S. niveus* mutants in the study of tyrosine and novobiocin biosynthesis

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Novobiocin is an aromatic, hydrophobic antibiotic produced by *Streptomyces niveus* ATCC19793 and *Streptomyces spheroides*, its site of action being the ATPase sub-unit of DNA gyrase.

This communication reports the isolation and characterisation of mutants of *S. niveus* to be used in a study of the relationship between the expression of the primary metabolic pathway (shikimic acid pathway) leading to tyrosine biosynthesis and the secondary metabolic pathway which metabolises tyrosine into novobiocin.

Twelve novobiocin production-defective mutants (novo$^-$ mutants) have been isolated following NTG mutagenesis. These have been classified into cosynthesis groups and also complemented with four intermediates from the novobiocin biosynthetic pathway. The intermediates were Ring A, novobiocic acid (Rings A & B), novenamine (Rings B & C), and decarbamylnovobiocic. Resistance to novobiocin and the induction of resistance by the mutants has been examined in relation to these complementation groups.

A tyrosine-overproducing mutant has been isolated, which is resistant to the toxic analogue, m-fluorotyrosine. It has been characterised and used to gauge the relationship between tyrosine production and the novobiocin biosynthetic pathway.

85. Transformation and transfection of *Streptomyces niveus*

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Transformation of *Streptomyces* species has been reported for a wide range of strains, however the transformation of wild-type antibiotic producers has often proved more difficult. This paper reports the findings of studies undertaken to develop a transformation and transfection system for *S. niveus*, as part of a wider study on the regulation of the biosynthesis of novobiocin.

Initial studies attempted to transform *S. niveus* protoplasts with a range of plasmid vectors. Early

results showed no transformation with either pIJ101 derivatives (pIJ702, pIJ680, pIJ486) or SCP2 derivatives (pIJ941, pIJ940). Conditions for growth of substrate mycelia were varied in both time and composition of liquid media, with no success in transformation studies. Attempts to overcome restriction barriers through heat shock of protoplasts, or addition of carrier-DNA failed to stimulate transformation. Later studies, however, using large quantities of plasmid DNA (up to 200 μg) showed low level transformation of *S. niveus* protoplasts by pIJ702. Plasmid DNA was re-isolated and retransformed into *S. niveus*, giving increased transformation (up to 10^3 transformants/μg). Further experiments failed to yield increased transformation frequencies.

Studies have been carried out to investigate a plasmid found in *S. niveus*. This plasmid (PSN2) has been restriction mapped and its potential use as a cloning vector is being studied.

Transfection studies using φC31 derivatives (KC301, KC515) have been undertaken. Low level liposome mediated transfection has been observed in both wild-type and mutant strains of *S. niveus*. The use of the two vectors in cloning studies is being evaluated.

86. Two DNA sequences which determine novobiocin resistance in the novobiocin producing organism *Streptomyces niveus*

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Novobiocin is a clinically useful antibiotic which is produced from tyrosine in *S. niveus* and *S. spheroides*, and this antibiotic is used in soil isolation procedures to select for thermoactinomycetes. Novobiocin production and glucose utilization in fermentation cultures of *S. niveus* occurs after 96 hours incubation when citrate has been exhausted. The synthesis of this aromatic antibiotic is regulated by three mechanisms, (1) citrate catabolite repression, (2) phosphate repression, and (3) nitrogen repression. Mycelial resistance to novobiocin during fermentation increases from 25–50 μg/ml to approximately 300–500 μg/ml novobiocin. This increase in novobiocin resistance can be repressed by the addition of citrate to the fermentation culture implying that novobiocin production and resistance may be linked.

Three fragments of *S. niveus* chromosomal DNA which determine novobiocin resistance have been cloned into the vector pIJ702 in *S. lividans* 66. These recombinant plasmids have been designated pGL101, pGL102 and pGL103. The novobiocin resistance levels of pGL102 and 103 are 100 μg/ml and the resistance level of pGL101 is 50 μg/ml. Restriction endonuclease analysis of the three clones revealed that pGL102 and pGL103 were identical but cloned in different orientation, and that pGL101 was unique. This conclusion was confirmed by Southern hybridisation experiments where the plasmids were blotted against each other. Probe DNA from pGL102 showed significant DNA homology to pGL103 but not to pGL101. Southern hybridisation experiments were also performed using chromosomal DNA isolated from novobiocin producing and non-producing organisms and DNA probes from the resistance clones. The clones showed no sequence homology with *S. lividans* or *S. griseus* DNA (novobiocin non-producers) but they did show sequence homology with DNA isolated from *S. niveus* and *S. spheroides* (producers). Therefore the two novobiocin resistance determinants appear to be sequence specific to novobiocin producing organisms and to be separate gene functions.

87. The further characterisation of UV sensitive mutants of *S. coelicolor*

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A number of UV sensitive mutants of *S. coelicolor* have been isolated and genetically mapped by Harold and Hopwood (*Mut. Res.*, *10*, 427–438, 1970 and *ibid.*, 439–448, 1970) and found to fall into six linkage groups, named *uvs A*, *B*, *C*, *D*, *E* and *F*. A number of double mutants of various combinations were also constructed. Successful crosses involving these mutants showed no apparent recombination defects, but little further investigation into the nature of these mutants has been carried out.

In an attempt to define and characterise these mutations causing UV sensitivity we have adopted a number of approaches. Firstly, a representative series of UV sensitive mutants, including a *uvsD*, *uvsF* double mutant were tested against a number of mutagens including UV light, mitomycin C (which induces bulky lesions in DNA), methyl

methane sulphonate, and ethyl methane sulphonate, which both alkylate DNA. The result showed a pattern of cross sensitivity which suggests that certain of the mutants might be defective in repair replication. Evidence supporting this conclusion was obtained from the observation that cell free extracts of some of the mutants were unable to catalyse nick translation reactions. These particular *uvs* mutants, therefore, appear to have a deficiency similar to that of *polA* mutants in *E. coli.*

88. Development of a phage-based cloning system for *Streptomyces venezuelae*

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To study in *Streptomyces venezuelae* the regulation of gene expression and protein excretion on a molecular basis, we are constructing a cloning vector based on actinophage VWB. VWB is a temperate phage, producing small turbid plaques on *S. venezuelae.* As a prophage VWB integrates into the host chromosome as proven by DNA hybridization experiments. VWB-DNA (47·3 kb) has an extremely high G+C content of 77·2 mol per cent (based on Tm) and it is susceptible to many restriction endonucleases. A restriction map of VWB has been constructed.

To develop from VWB a cloning vector, a thiostrepton resistance gene together with a DNA fragment having a unique *Xba*I recognition site were cloned in VWB, resulting in VWB-04. To make VWB-04 more useful, additional cloning sites were inserted using a *Xba*I-*Dra*I-*Xho*I linker. Although this construct (VWB-05) contained 1·19 kb supplementary DNA compared to the original VWB, the phages remained stable and could lysogenize *S. venezuelae* rendering the lysogens thiostrepton resistant.

To estimate the packaging capacity of VWB, DNA fragments of different sizes were cloned into VWB-04. Results indicated that VWB phage heads could stably package at least 107·6 pct compared to the original phage DNA.

89. Organization and expression of streptomycin production genes in *S. griseus*

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Eight to ten putative streptomycin (SM) production genes have been analysed so far by nucleotide sequencing of DNA cloned from *S. griseus* N2-3-11. Three genes, *aphD* (SM resistance), *strR* (positive regulator) and *strB* (amidino transferase). Detailed transcription studies were carried out in wildtype and mutant strains. A model for the regulatory mechanism exerted by the putative *strR* gene product will be presented.

A second SM resistance gene, *aphE*, not clustered with known production genes was also investigated. Both resistance genes code for unrelated SM phosphotransferases, *aphD* for APH(6) and *aphE* for APH(3″). The relationship to other antibiotic and protein kinases will be discussed.

90. Secretory expression of interleukin-2 by *Streptomyces lividans*

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Transport of proteins through membranes is mediated by the so-called signalpeptide, the role of the protein to be transported and of the membrane during this process is largely unknown. Streptomyces secrete a variety of proteins, one of them is tendamistat, produced by *Streptomyces tendae.* This inhibitor of mammalian α-amylase (EC 3.2.1.1.) is a peptide of 74 amino acids, whose gene was isolated and sequenced (Koller *et al.*, unpublished). The DNA sequence reveals a leader consisting of 30 amino acids whose structure resembles that of the known signalpeptides.

For testing the ability of *Streptomyces lividans* to secrete a heterologous protein, we made a fusion between the DNA sequence of the tendamistat leader and the chemical synthesized (Engels, J. W., Uhlmann, E., Wengenmeyer, F., Müllner, H., Winnacker, E. L., Mertz, R. and Okazaki, H., 1985, DE 3419995) nucleotide sequence coding for the mature part of interleukin-2. Therefore an *Apa*I recognition site was introduced into the DNA of the signalpeptide by using the "gapped duplex"

method for site directed mutagenesis. With the help of an oligonucleotide linker the DNA-sequence of interleukin-2 could now be ligated with the leader of the tendamistat gene. The construct was verified by Sanger-sequencing and subcloned into a pIJ702 derivative. After transforming *Streptomyces lividans* TK24, aliquots from supernatants of positive clones were tested for interleukin-2 activity *via* an interleukin-2 dependent cell line, whereby two clones showed interleukin-2 activity in the range of 20 μ/ml.

91. Site-specific mutagenesis of the α-amylase inhibitor tendamistat

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Tendamistat, a 74 amino acid peptide was isolated from *S. tendae* and is a powerful inhibitor of mammalian α-amylases (Vértesy *et al.*, *Eur. J. Biochem.*, *141*, 501–512, 1984).

Based on the three dimensional structure of tendamistat (Pflugrath *et al.*, *J. Mol. Biol.*, *189*, 383–386, 1986) we started a program of site specific mutagenesis of this inhibitor. The known gene sequence (Koller *et al.*, unpublished) was incorporated into the M13 genome. By the "gapped duplex" method (Kramer *et al.*, *Cell*, *38*, 879–889, 1984) using two mutagenic primers aiming at replacing the disulfide bridge from position 11 to 27 by two alanines we constructed the desired mutations. With "molecular dynamics" computer program (AMBER, Weiner *et al.*, *J. Am. Chem. Soc.*, *106*, 765–784, 1984) the replacements were calculated within the frame work of the X-ray structure. Following the mutagenesis protocol both mutagenic primers were annealed simultaneously. After fill in the transfection phage plaques were screened.

The correct exchange TGC(11)→GCG and TGT(27)→GCT could be verified by DNA-sequencing according to Sanger's procedure. After reintroducing the mutated gene into the expression plasmid pIJ702 and transforming into *S. lividans* we tested for the synthesis of this modified tendamistat. By a plate assay (iodine-starch) (Koller *et al.*, 3. *Eur. Congress on Biotechnology*, Vol. III, 273–278, 1984, Verlag Chemie, Weinheim), the inhibition of added mammalian α-amylase by this peptide could be shown. We hypothesize that this peptide probably still has a similar tertiary structure and can be transported into the culture medium.

92. Mutational cloning in *Streptomyces coelicolor* A3(2) using a multicopy ts replication plasmid pMT660

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Plasmid pMT660 (Birch and Cullum, *J. Gen. Microbiol.*, *131*, 1299, 1985) is a ts-replication mutant of pIJ702 with a restrictive temperature of 39°C. Chromosomal DNA fragments can be readily cloned into pMT660. Selection for the plasmid marker (thiostrepton resistance) at the restrictive temperature allows identification of recombinant plasmids which have become inserted into the chromosome *via* homologous recombination between the cloned fragment and the host chromosome. The suitability of pMT660 as a general mutational cloning vector will be discussed.

93. Cloning of DNA sequences from *Streptomyces longisporoflavus* which confer increased resistance to the ionophore antibiotic tetronasin in *Streptomyces lividans*

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S. longisporoflavus produces the polyketide polyether antibiotic tetronasin (formerly ICI 139603). *S. lividans* is able to grow in the presence of tetronasin, but sensitivity to the antibiotic is enhanced by addition of Na^+ and Ca^{2+}.

A genomic DNA library of a high tetronasin-producing strain of *S. longisporoflavus* was constructed using the positive selection vector pIJ699 (Kieser and Melton, *Gene*, 1988, in press) and transformed into *S. lividans* TK64. When recombinants from the library were screened by selection on media containing 10 μg ml^{-1} tetronasin in the presence of 0·2 M NaCl, two resistant colonies

were identified. The recombinant plasmids were isolated from these colonies. They both conferred increased resistance to tetronasin when retransformed into *S. lividans* TK64.

94. Expression and control of expression from the *tac* promoter in *Streptomyces*

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Using pIJ486 (Ward *et al.*, *Mol. Gen. Genet.*, *203*, 486–478, 1986), two bifunctional vectors for *E. coli* and *Streptomyces* have been constructed. They contain the promoters *lac* (pGLW49) and *tac* (pGLW50) just upstream of a promoter-less kanamycin phosphotransferase gene (*aph*II) gene (*aph*II).

Both vectors conferred resistance to kanamycin in *E. coli*, but only the vector carrying the *tac* promoter conferred resistance to kanamycin in *Streptomyces lividans*.

A third bifunctional vector, pGLW51, was constructed. It contained not only the *tac* promoter but also a gene coding for the *lac* repressor ($lacI^Q$).

Clones of *E. coli* and *Streptomyces* containing pGLW51 showed elevated levels of resistance to Kanamycin in the presence of IPTG. This indicates that regulation of the *tac* promoter by the *lac* repressor was occurring in *Streptomyces* as well as in *E. coli*.

*Aph*II enzyme assays of crude extracts of cultures of *E. coli* and *Streptomyces* containing pGLW49, pGLW50 and pGLW51 are being performed in parallel with S1 mapping of the *aph*II mRNA's in both species.

95. Molecular analysis of the oxytetracycline-resistance gene, *otc*1, from *Streptomyces rimosus*

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The oxytetracycline (OTC) resistance gene *otc*1 is located at one end of a gene cluster for production of the antibiotic. The gene product confers resistance to OTC on the host's ribosomes, although this is not due to a covalent modification of the ribosomal proteins or rRNA.

The gene has been sequenced. It displays the usual triplet bias of streptomycete coding regions. The size of the open reading frame deduced from the DNA sequence is in good agreement with the data from *in vitro* transcription/translation experiments. The gene product has significant amino acid homology with EF-TU from *E. coli* and *tet*M from *Streptococci*.

The transcription pattern of the gene is complex. It has two tandem promoters and several strong promoters point antisense to the *otc*1 transcript.

96. Proline metabolism in *Streptomyces coelicolor*

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Streptomyces coelicolor is able to utilise proline as both carbon and nitrogen source for growth. In this communication we report the isolation of several mutants defective in proline metabolism. These mutants were isolated after mutagenesis and exposure to analogues of proline. Of the twelve different proline analogues tested, only a few proved useful.

The activities of the proline transport enzyme, proline permease, and that of the proline catabolic enzymes, proline oxidase and pyrroline-5-carboxylate dehydrogenase, were detected in the parent J802. However, mutant PTM44f which was resistant to azitidine-2-carboxylate (AC), was found to be defective in both the proline transport and catabolic enzymes. Other mutants were defective in the proline permease activity only.

Some of these mutants, which are resistant to toxic proline analogues in growth medium of low salt concentrations exhibit enhanced sensitivity to the same analogues and growth medium with increased NaCl concentrations. This observation implies that a second proline uptake system, which functions only under elevated osmotic conditions, may be present. We will report the progress of our genetic analysis of these mutants in our poster.

97. Analysis of chromosomal deletions and amplifications in *Streptomyces glaucescens*

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Streptomycetes exhibit an unusually high level of genetic instability, affecting a variety of genes, several of which are known to be chromosomally located. One aspect of this is the occurrence of gross chromosomal rearrangements including extensive sequence amplification and deletion. In *S. glaucescens* this phenomenon has been investigated predominantly with respect to the loss of tyrosinase activity and streptomycin resistance, both of which arise due to extensive deletion (Hintermann *et al.*, *Mol. Gen. Genet.*, *200*, 422–432, 1985 and *196*, 513–520, 1984). Amplifications are frequently detected in such double mutant strains, these show a wide variation in the sequence amplified and the extent, stability and copy number of the reiteration (Hasegawa *et al.*, *Mol. Gen. Genet.*, *200*, 375–384, 1985). Cosmid bank analyses have permitted the mapping of the amplifications and deletions to a single extensive chromosomal region. All amplifiable unit DNAs (AUDs) occur within a 100 kb region and may be simple or complex in nature. The deletions all have a common end which is situated approximately 770 kb from the AUD region. They extend uni-directionally towards the AUD region, the longer ones covering the entire distance and terminating adjacent to, or in the neighbourhood of the AUDs. The deletions span, maximally, the streptomycin resistance gene (270 kb), the tyrosinase gene (350 kb) and finally up to the AUDs, a total of 770 kb. Numerous shorter deletions are also detected which generate single marked strS strains or wild-type-like strains, ending before the streptomycin resistance gene. The amplifications may be generated as a response to the deletion, either as a protection measure, as a result of the joining of previously distinct sequences or the loss of some undefined gene product under the deletion.

98. The mel C-operon from *S. glaucescens*: characterisation of the promoter

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The gene from *S. glaucescens* coding for an inducible tyrosinase was cloned using the vector pIJ41 and the melanin negative *S. lividans* strain TK23 as a host. The mel C gene is part of a 2·3 kb transcript and is flanked by two other open reading frames of unknown function. The mel C operon is regulated on the transcriptional level as shown by northern hybridisation with RNA from induced and uninduced cells.

The start point of transcription was determined by mapping with nuclease S1, mung bean nuclease and by primer elongation. The promoter shows no homology with the postulated *E. coli*-like Streptomyces promoter consensus sequence. A deletion set of the upstream region was constructed in order to define sequences important in regulation of the transcription. The partially deleted promoters could be grouped into four classes dependent upon expression and regulation. Cell free extracts of *S. glaucescens* and *S. lividans* contain a putative regulatory protein which binds upstream of the promoter as was shown with DNAseI footprint experiments and by gel retardation assays.

99. Analysis of amplified DNA in *S. glaucescens*

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The *S. glaucescens* chromosome in common with other *Streptomyces* contains regions which are prone to rearrangement. Deletions affect primarily genes of the secondary metabolism and are often found in association with amplifications. In *S. glaucescens* the tyrosinase(melC)- and the streptomycin phosphotransferase(sph)-genes have been characterized as "unstable" and subject to high frequency deletion. Amplification of sequences ranging from a few to over 30 kb occurs in this strain in one particular 100 kb region of the chromosome.

The amplifiable units of DNA (AUD) are present as single copies and no two were found to be identical.

Northern blot experiments showed that no detectable transcript could be found from a stable amplified sequence (ADS), which is present as tandem repeats in about 500 copies per chromosome. Sequencing of the ends of the amplifiable unit showed no repeated structure which would be responsible for reiteration.

In order to analyse the structure of the amplification/deletion-Junction, the high copy number was drastically reduced by protoplasting

and regeneration of the mutant strain. Analysis of the deamplified DNA-structure showed that only a few units (5-8) were still present, but that additionally a secondary deletion had been induced.

Sequencing data revealed an imperfect palindromic sequence at the endpoints of the secondary deletion.

100. Deletion forms of pMEA100

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The plasmid pMEA100 has been found in an actinomycete *Amycolatopsis mediterranei* (*Nocardia mediterranei*) (Moretti *et al.*, *Plasmid*, *14*, 126-133, 1985). It occurs both in a chromosomally integrated form and in the free state. The integrated form of pMEA100 can be excised precisely from the chromosome. After transfer into the plasmid-free *A. mediterranei* strain, pMEA100 reintegrates site specifically into its original chromosomal locus (Madon *et al.*, *Mol. Gen. Genet.*, *209*, 257-264, 1987).

After conjugal transfer of pMEA100 from pMEA100-containing into plasmid-free cells, both the integrated and the free form were found in the recipient strain. However, the analysis of the cccDNA after crossing showed, that the original element of pMEA100 is present together with different deletion derivatives of it.

It has also been shown, that a subculture of the original *A. mediterranei* strain LGBA3136 contains in addition to the integrated form of pMEA100, numerous deletion derivatives of the original plasmid.

The Southern hybridization analysis of these deletion forms showed, that none of them contains the integration region of the pMEA100 plasmid.

Analysis of the cccDNA in different *A. mediterranei* colonies from one population showed, that the distribution of the pMEA100 deletion forms is not uniform. About 60 per cent of the analyzed samples contained only pMEA100 in the cccDNA fraction. The rest of colonies showed in addition varying amounts of pMEA100 deletion forms (1 to 4). In no case could a deleted plasmid be detected without the presence of undeleted pMEA100.

The transformation of *A. mediterranei* cells with the plasmid preparation containing deletion froms of pMEA100 led to the isolation of the *A. mediterranei* cells containing only a single pMEA100 deletion form.

Studies are presently in progress to determine the suitability of such deletion derivatives of pMEA100 for the construction of a vector system in *A. mediterranei.*

101. Excision of pIJ408 from the chromosome of *Streptomyces glaucescens* and its transfer into *Streptomyces lividans*

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Conjugative *Streptomyces* plasmids are known to cause lethal zygosis when a spore or a mycelial fragment of a streptomycete strain carrying a conjugative plasmid grows in the presence of an excess of individuals lacking the plasmid. This phenomenon is also known as pock formation as it leads to an area of retarded development, a so called "pock".

The conjugative plasmid pIJ408 was found in *S. lividans* 66 after its mating with *S. glaucescens* wild type strain (Hopwood *et al.*, *Plasmid*, *11*, 1-6, 1987), by its ability to form pocks on transfer to pIJ408 free *S. lividans* 66 cells.

pIJ408 exists only as an integrated DNA sequence in the chromosome of *S. glaucescens* GIA000, from which it can be transferred into *S. lividans* 66. Southern hybridization showed that the plasmid exists in *S. lividans* 66 both as an integrated sequence in the chromosome and as a free autonomously replicating form. Strains were isolated which contained the plasmid in both states, in integrated from only and in free form only. The excision of pIJ408 from the chromosome of *S. glaucescens* wild type is likely to occur site-specifically.

In order to understand the mechanism of excision and integration of pIJ408, the DNA region of the plasmid involved in integration was subcloned and the integration point localized by restriction analysis and Southern hybridization experiments. The flanking regions of the chromosomally integrated form of *S. glaucescens* GLA000 were identified with the help of a λ gene bank.

DNA sequencing of the integration regions of pIJ408 and of the flanking segments of its integrated form has been performed.

102. The characterization and cloning of the *Not*I restriction-modification system

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*Not*I, a Type II restriction-modification system from the actinomycete *Nocardia otitidiscaviarum*, recognizes the sequence GCGGCGC.

We are currently purifying and characterizing the proteins of this system. The endonuclease, which cleaves at GC▼GGCCGC, has been sized by gel filtration and estimated to be approximately 85,000 daltons. The NotI methylase protein has been partially purified and we are in the process of determining its size as well as the site and type (N-4 or C-5) of modification it provides. The *N. otitidis* genomic DNA has been purified and analyzed by HPLC analysis for base composition and for the presence of modified bases.

Two approaches are being used to clone the *Not*I system. First genomic libraries constructed in *E. coli* are being selected for methylase expression. Second, genomic libraries will also be made and selected in *Streptomyces lividans*. In addition to selection for methylase expression, we also will be selecting the libraries with phage. This approach has been successfully used in cloning the *Sal*I restriction-modification system (M. R. Rodicio and K. Chater, pers. comm.).

103. Molecular analysis of a DNA sequence from *Streptomyces alboniger* which carries the genes *pac* and *dmpm* of the puromycin biosynthetic pathway

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The genes *pac* and *dmpm* encoding puromycin N-acetyl transferase and O-demethylpuromycin O-methyl transferase, respectively, were found to be present in a 2·4 kb *Sal*I DNA fragment of the *S. alboniger* genome. Both subcloning and coupled transcription-translation experiments indicate that *pac* and *dmpm* are include in different transcriptional units. The *pac* promoter was isolated in the promoter-probe vector pIJ486 and its transcription start point determined by high resolution S1-mapping. The nucleotide sequencce of a 1·1 kb *Sal*I-*Bam*HI DNA subfragment encoding the genes *pac* has been deduced. About 60 per cent of the remaining 1·3 kb *Bam*HI-*Sal*I DNA region, putatively encoding the *dmpm* gene, has been sequenced so far.

104. Cloning genes from *Streptomyces tendae* involved in nikkomycin biosynthesis

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Nikkomycins belong to the nucleoside peptide antibiotics and act as competitive inhibitors of chitin synthetase. To isolate nikkomycin biosynthetic genes shotgun experiments were performed using pIJ699 as vector (kindly provided by T. Kieser) and size fractioned chromosomal DNA of the wild type partially digested with *Bam*HI. The ligation mixture was transformed into a nonproducing mutant of *S. tendae*. Among 3000 transformants screened for antifungal activity one clone carrying a 8·4 kb insert was biologically active. In liquid medium this clone produced about 50 mg/l nikkomycin K_X and 200 mg/l nikkomycin C_Z/C_X as judged by HPLC analysis and UV spectra of the eluted peaks. Restriction analysis of the insert DNA, Southern hybridisation studies with digested chromosomal DNA's of different mutants of *S. tendae* and other *Streptomyces* species and complementation experiments will be reported.

105. The molecular biology of *S. thermonitrificans* ISP5579–a thermophilic streptomycete

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Streptomyces thermonitrificans has been chosen to test the feasibility of antibiotic production in a heterologous host at elevated temperature. At higher fermentation temperatures several process advantages are expected, the most important

of which is the reduced cooling requirement. This may lead to a considerable saving in energy costs at industrial scale.

The development of an endogenous cloning system for a thermophilic streptomycete, such as *S. thermonitrificans*, is required for the optimal exploitation of their biotechnological potential and for a detailed examination of their molecular biology so that they may be compared with their mesophilic relatives.

A summary of the past three years' work on the biology of *S. thermonitrificans* will be presented. The work includes the development of a plasmid transformation system using both low and high copy number vectors, the isolation of a thermotolerant actinophage, analysis of restriction between *S. thermonitrificans* and *S. lividans*, the identification of a plasmidogenic transposable element which resides in the chromosome of *S. thermonitrificans* and the heterologous expression of an antibiotic biosynthetic pathway in *S. thermonitrificans* at temperatures above the growth range of the producing mesophilic organism.

106. Amplification of DNA by continuous selection in streptomyces

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Amplifiable DNA sequences are observed in many Streptomycete species (reviewed by Cullum *et al.*, In: *Biotechnol. Genet. Eng. Rev.*, *4*, 59, 1986). DNA amplification may occur spontaneously or when cells are exposed to stress, as in the presence of an antibiotic. Amplifiable sequences lend themselves to the construction of amplification vectors and the investigation of new techniques for the selection of mutants carrying amplified DNA is important in their development. In a number of cases step-wise increasing of antibiotic resistance has proved suitable for the selection of amplified DNA (Horneman *et al.*, *J. Bacteriol.*, *196*, 2360, 1987; Flett *et al.*, *Mol. Gen. Genet.*, *207*, 499, 1987).

A method for the continuous selection of highly antibiotic resistant mutants of Streptomyces, adapted from that described by Brown and Oliver (*Eur. J. Appl. Microbiol. Biotechnol.*, *16*, 119, 1982), has been developed. Selection is achieved by controlling growth in continuous culture by antibiotic additions to the culture vessel. The rate of antibiotic addition is directly proportional to the metabolic activity of the culture as measured by carbon dioxide evolution. Feedback loops and continuous monitoring of antibiotic concentrations within the vessel are made possible by the use of a microprocessor controlled fermenter.

A mutant of *S. lividans* TK64 highly resistant to chloramphenicol has been selected for using the above technique. Investigation of genomic DNA by Southern blot analysis has revealed the presence of a DNA amplification in this strain. The amplified DNA is derived from a region of the chromosome where it is believed the chloramphenicol resistance determinant lies. Work to characterise the amplified DNA is now in progress.

107. Analysis of large deletions associated with amplifiable DNA regions in *Streptomyces lividans*

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Genetic instability has been demonstrated in many *Streptomyces* species where specific mutants occur spontaneously at a frequency greater than 0·1 per cent of spores. *Streptomyces lividans* yields chloramphenicol sensitive (Cml^S) mutants at high frequency (0·1–1 per cent) and the Cml^S mutants are very unstable giving arginine auxotrophs (Arg^-) at frequencies of 5–25 per cent.

The Cml^S mutants have large DNA deletions which presumably cover the Cml^r gene (Flett and Cullum, *Mol. Gen. Genet.*, *207*, 499, 1987). In the Arg^- mutants a particular 5·7 kb chromosomal DNA sequence is highly amplified and deletions (including *argG*) are found to one side of this sequence (Altenbuchner and Cullum, *Mol. Gen. Genet.*, *201*, 192, 1985).

Recently we have been studying the size of the deletions and the new junction bands arising from them. We have investigated the extent of the deletions by probing a cosmid bank of the parental *S. lividans* strain with total DNA from a Cml^S Arg^- mutant. Clones which did *not* hybridise were presumed to lie in a deletion and were studied further.

The new junction bands formed by the Arg^- deletions were examined by Southern blotting with the 5·7 kb amplified fragment as probe. These

experiments then led on to the cloning of certain junction bands. Our results demonstrate that the deletions are very large and can vary in length thus giving rise to different novel junction fragments.

108. Regulation of α-amylase expression in *Streptomyces albus* G

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Streptomyces albus G produces two α-amylase activities of 67 k and 55 k during growth on media containing starch (Andrews and Ward, *Biochem. Soc. Trans.*, *15*, 521, 1987).

Induction studies using a shift from a non-inducing carbon source to starch-containing media show a slow onset of amylase production of the order of several hours. Experiments to find the true inducer were carried out.

The effect of various carbon sources on the induction and repression of the amylase activities was studied and shows that the *S. albus* G amylases are regulated in a different manner to other streptomycete amylases.

109. The occurrence of extracellular serine proteases in several *Streptomyces* strains

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Streptomycetes produce a vast array of extracellular metabolites. Hydrolytic enzymes are present in this secreted material and enable *Streptomycetes* to break down and utilise complex organic materials present in soil. Among these are a range of proteolytic enzymes whose occurrence has already been investigated in several *Streptomyces* species including *S. griseus* and *S. rimosus*.

S. griseus strain k_1 proteolytic enzymes are produced commercially and are marketed under the name Pronase. At least twelve different enzymes have been characterised so far in Pronase, including the serine proteases *S. griseus* trypsin (SGT), *S. griseus* proteases A and B (SGPA and B) and *S. griseus* subtilisin (SGS). (Nomoto *et al.*, *J. Biochem.*, *48*, 453, 593, 906, 1906; Trop and Birk, *J. Biochem.*, *116*, 19, 1970).

S. rimosus has also been shown to produce a trypsin-like enzyme and an alkaline serine protease with activity against aromatic amino acids (Renko *et al.*, *Eur. J. Appl. Microbiol. Biotechnol.*, *11*, 166, 1981).

The aim of this study was to look at the occurrence and relative amounts of serine proteases of different specificities produced in overnight cultures of twelve *Streptomyces* species.

110. Identification and isolation of *S. coelicolor* A3(2) hsp 70 gene

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Heat shock response was observed for the first time in *Drosophila* and then in all organisms, from prokaryotes to man; it is also induced by other stimuli such as exposure to ethanol, DNA damaging agents, oxidative phosphorylation inhibitors and oxiding agents (Lindquist, *Ann. Rev. Biochem.*, *55*, 1151, 1986).

Our poster presents results about the isolation of hsp 70 gene from a *S. coelicolor* genomic library.

In *Streptomyces coelicolor* A3(2), a temperature shift-up (from 30 to 37°C) induces the synthesis of, at least, two proteins (~70 and 62 Kd). This response occurs either on substrate or on aerial mycelium. An interesting feature is that, in 21–25 hr old cultures, (just after mycelium substrate formation and immediately before aerial hyphae production), these two proteins are present in a relatively large amount also at 30°C. A *bald* mutant (*bld*FI) which doesn't differentiate, shows no induction of heat shock proteins.

In order to isolate hsp genes, a *S. coelicolor* genomic library was constructed in EMBL 3 vector. By hybridization with *H. capsulatum* hsp 70 probe, 10 positive plaques were observed; the recombinant phage DNA, *Sal*I digested, hybridized with the probe.

The characterization of some *Sal*I inserts, subcloned in pUC 19 vector, is also presented.

111. Cloning of a DNA region involved in *Steptomyces* secondary metabolism

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As it is known, the *S. coelicolor* colony has very complex structures. However its genetic analysis has been carried out obtaining *bld* and *whi* mutants (Chater K., *Microbial Development*, CSH, 89, 1984).

In our lab, a *bld* mutant (*bld*FI) was isolated after NG treatment. This mutant, that cannot be referred to any of the known classes, doesn't produce actinorhodin and undecylprodigiosin antibiotics.

Our poster presents results about the cloning of a DNA region which seems to be involved in the secondary metabolism of *Streptomyces*.

In shotgun cloning experiments (in order to isolate the gene complementing *bld*F1 mutation), we identified a clone which, very early, produces large amounts of actinorhodin. The plasmid DNA (pSCb1I) extracted from this clone induced actinorhodin synthesis in *S. lividans* 66 and in the *bld* mutant strains J1700 and J774. An interesting feature is that the pSCb1I induced the production of aerial mycelium and spores in J1700 strain.

Further experiments are being carried out to understand the pSCb1I role in *Streptomyces* secondary metabolism.

112. Cloning of the histidine biosynthetic genes of *Streptomyces coelicolor*

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The structural organization of the genes involved in the biosynthetic pathway for the amino acid histidine in *S. coelicolor* is intermediate in complexity between that of enterobacteria where all the genes are clustered in an operon and that of lower eukaryotic cells where each gene resides on a distinct chromosome. We have begun to investigate the structure and expression of the *his* genes in *S. coelicolor.*

A genomic bank was constructed by partial *Sau*3AI digestion of the chromosomal DNA and cloning in the *E. coli* expression vector plasmid pUC12. Clones carrying *his* genes were isolated by double selection for antibiotic resistance and complementation of *E. coli his* auxotrophic strains on minimal medium. One such plasmid (pSCH1) contains a DNA insert of 3400 base pairs of which a detailed restriction map has been established. The DNA insert hybridizes specifically to *S. coelicolor* genomic fragments in Southern blots. The DNA fragment contains more than one *his* cistron since it is able to complement several *E. coli his* auxotrophic strains and therefore corresponds to the *his* gene cluster of *S. coelicolor*, possibly organized as a polycistronic unit. These *S. coelicolor his* genes appear to be expressed in *E. coli* from their regulatory elements since complementation is observed upon cloning of the DNA fragment in both orientations.

113. Efficient plasmid transformation of industrial strains of *S. pristinae spiralis*

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First attempts to transform industrial strains of *S. pristinae spiralis* with plasmid pIJ702 yielded not more than 10^2 to 10^3 transformants per ug of plasmid DNA. When transformation was carried out with plasmid DNA isolated from *S. pristinae spiralis* transformants the frequencies obtained were not increased.

We determined and carefully examined parameters which may influence the efficiency of transformation. Among them, protoplast formation and protoplast regeneration appeared to play a major role. Definition and standardization of the culture before protoplast formation and of the drying of the regeneration plates resulted in an increase of the frequencies of transformation of about 1000 fold. Frequencies of $5 \cdot 10^5$ to 10^6 transformants per μg of pIJ702 DNA are now routinely obtained.

114. Mutational cloning in *Streptomyces ambofaciens*

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Spiramycin is a 16 membered macrolide antibiotic produced by *Streptomyces ambofaciens.*

A c^+ att P deleted derivative, KC515, of the *Streptomyces* temperate phage ϕC31 was used to clone fragments of genes involved in Spiramycin biosynthesis.

Phages carrying 2-4 kb *S. ambofaciens* genomic DNA inserts into Thiostrepton resistance gene (Tsr) were selected by protoplasts transfection of *S. lividans* TK21. After replica plating onto spiramycine producing strain spore layers of 250 insert-directed transductants were selected (Viomycin resistant-Thiostrepton sensitive lysogens). 10 per cent of those transduced strains are non-producing mutants and 1 per cent are overproducing mutants. The presence of the integrated recombinant phage in the genomic DNA was confirmed by non radioactive hybridization.

The viability of this technique for the isolation of spiramycin biosynthesis genes will be discussed.

115. Potential nonsense suppressors in *Streptomyces lividans*

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The existence of various nonsense suppressor mutations has been useful in *E. coli* genetic studies. No such mutations have yet been described in *Streptomyces.* To obtain this type of mutation in the latter species, amber mutations were first constructed by *in vitro* mutagenesis of four antibiotic resistance genes encoding antibiotic modifying enzymes conferring resistance respectively to neomycin, apramycin, hygromycin and bialaphos. Supression of these mutated genes was checked and found in a range of *E. coli* amber suppressors.

Derivatives of pIJ699, an *E. coli*/*Streptomyces* shuttle plasmid (Ward *et al., Mol. Gen. Genet., 203,* 468, 1986) were constructed, containing various combinations of pairs of these genes.

After transformation of *S. lividans* mutagenised with nitrosoguanidine, expression of the mutated genes was selected. Clones expressing simultaneously both resistances were found and crude extract analysis revealed the presence of the expected modifying enzymes.

Those clones are candidates for amber suppressors in *Streptomyces*; further analyses are in progress.

116. Cloning and analysis of two iron-regulated genes of *Streptomyces pilosus* involved in desferrioxamine B biosynthesis

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The desferrioxamines are a class of siderophores produced primarily by genera of the Actinomycetales. Siderophores play an important role, together with their receptor molecules, as high affinity iron transport systems. Cloning of genes from *S. pilosus* involved in desferrioxamine synthesis was carried out in order to study the genetic control and regulation of this iron transport system in streptomycetes.

By selfcloning experiments using a blocked mutant of *S. pilosus* and the plasmid vector pIJ943 a DNA fragment of 4·5 Kb was identified which complemented two steps of the pathway (Schupp *et al., Gene,* in press). Expression of the first biosynthetic enzyme, lysine decarboxylase (Schupp *et al., FEMS Microbiol. Lett., 42,* 135, 1987), was detected with the cloned fragment and a 2·8 Kb subfragment. It was also possible to express and identify two gene products of the cloned DNA in *E. coli.* One of these proteins is possibly the lysine decarboxylase.

Northern blotting and S1 mapping experiments showed that the two cloned genes of the pathway are transcribed as one polycistronic m-RNA and that the level of transcription is regulated by iron. Using the promoter probe plasmid pIJ487 (Ward *et al., Mol. Gen. Genet., 203,* 468, 1986) a promoter-active fragment was detected. The location of this fragment together with the analysis of the transcript allowed to define the region containing the promoter and transcriptional start site for the two investigated genes of siderophore biosynthesis. DNA sequence analysis of this region using the dideoxy method was performed. A sequence upstream from the transcriptional start point was found with significant homology to the −10 and −35 consensus sequences of *E. coli.* 79bp downstream from this promoter region a long open reading frame was identified coding most probably for the lysine decarboxylase.

117. Dispersed growth of *Streptomyces* in liquid cultures

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Growth of the majority of *Streptomyces* in liquid cultures results in pellet formation. These pellets are essentially microcolonies composed of a heterogeneous mixture of cells, some of which may have become nutrient starved. These cultures are therefore complex and difficult to study at a physiological level. The present work describes a method to obtain dispersed growth of *Streptomyces* which will facilitate investigation of these organisms.

118. Genetic instability in *Streptomyces lividans*

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Many strains of Streptomycetes are genetically very unstable. Typically, these strains alter characteristics such as aerial mycelium and spore formation, antibiotic and pigment production at high frequencies. In addition many traits used in numerical taxonomy differ significantly among several variants and their corresponding wildtype strains.

In order to understand the molecular mechanisms leading to genetic instability, *Streptomyces lividans* was analysed.

Morphological variants have frequently altered pigmentation and give rise to chloramphenicol-sensitive (clm^s) variants which segregate further to argG (argininosuccinate synthase) negative variants. If, however, selection is kept for chloramphenicol resistance, tetracycline-sensitive (tet^s) strains can be isolated at high frequency which segregate further to Ntr-variants. These variants still contain glutamine syntethase which can be modified by adenylation, but is no longer positively regulated.

Unstable genes encoding argininosuccinate synthase, chloramphenicol- and tetracycline-resistance have been cloned. Using gene libraries, DNA regions surrounding the various unstable determinants have been characterized. Comparative hybridizations revealed that most argG-variants arise by deletion of at least 60 kb including the argG-gene; chloramphenicol-sensitive, argG-variants delete at least an additional 15 kb of DNA including the determinant for chloramphenicol resistance. Tetracycline-sensitive variants lose at least the determinant encoding tetracycline-resistance, and Tet^s Ntr-strains carry an additional deletion a 4·4 kb amplifiable unit (see below).

In addition to deletion events, amplification of specific sequences is likely to occur. Within *S. lividans* two amplifiable units (AUD) have been characterized. The 6·8 kb AUD is present in one copy in TK23 and has a tandem repeated structure in the wildtype genome. The 4·4 kb AUD resides only as one copy in the wildtype genome. Both AUDs are flanked by direct repeats. If present as duplicated structure amplification is a frequent event. Thus, most argG, clm^s strains deriving from the wildtype strain contain in addition to the extensive deletions (see above) 300–500 copies of the 6·8 AUD in tandem array. In the wildtype genome this element is located 25 kb away from the argG gene.

Amplification of an AUD-element occurs rarely when it is present in one copy. Thus, amplifications of the one 4·4 kb AUD copy in the wildtype and the 6·8 kb AUD copy in the TK23 genome are rare events. If, however, a single-copy AUD is converted to a deplicated structure, amplification occurs at high frequency. The 4·4 kb and the 6·8 kb amplified DNAs encode no known functions. However, upon selection for high level chloramphenicol resistance its determinant becomes amplified.

In order to analyse the large deletions, conditions have been optimized to analyse total DNA of various *Streptomyces* strains with "pulsed-field-electrophoresis". Using optimized conditions high molecular weight DNA (>1·500 kb) can be characterized from most strains. In contrast, from *S. lividans* wildtype and its variants only relatively low molecular weight DNA (250 kb–20 kb) can be identified after electrophoresis in a "pulsed-field". Since the action of nucleases can be excluded, we assume the occurrence of specific DNA modifications leading to breaks during electrophoresis. Specific base modifications (for details see abstract 40) have recently been characterized within amplified DNA. The possible significance of these findings for the genetic instability is presently under investigation.

119. Organisation and nucleotide sequence analysis of a ribosomal RNA gene cluster from *Streptomyces ambofaciens*

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Streptomyces ambofaciens genome contains four copies of the ribosomal RNA gene cluster. These copies are called rrnA, B, C and D. The complete nucleotide sequence of rrnD has been determined. The structural genes are arranged in the order 16S–23S–5S and are tightly linked. These genes present a strong homology with other procaryotic rRNA genes. The extremities of the coding sequences have been determined by comparison with other rRNA sequences. The mature rRNAs are predicted to contain 1528, 3120 and 120 nucleotides respectively for the 16S, 23S and 5S rRNA.

Proceeding from these data, secondary structure models of S. ambofaciens rRNAs are proposed, based on those existing for bacterial rRNAs. The 23S rRNA is to our knowledge the longest among sequenced procaryotic 23S rRNA. When compared to other 23S rRNA it shows insertions at positions where they are also present in some archaebacterial and in eucaryotic large rRNA.

Putative transcription initiation and termination signals have been located. The corresponding primary transcript contains the 16S, 23S and 5S rRNAs plus flanking regions. This putative primary transcript has been folded into a secondary structure, and sequences possibly involved in maturation have been located.

The G+C content of the rRNA gene cluster is about 57 per cent which is low for *Streptomyces* which have an overall G+C content of about 73 per cent.

120. Isolation, nucleotide sequence and promoter study of an aminocyclitol acetyltransferase gene from *Streptomyces rimosus* forma *paromomycinus*

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A gene (*aacC5*) encoding an aminocyclitol 3-*N*-acetyl-transferase (AAC(3)-V) from *Streptomyces rimosus* forma *paromomycinus* NRRL 2455 was cloned in the *Streptomyces* plasmid pIJ702 and expressed in *Streptomyces lividans* 1326. A 1495 base pairs DNA segment encoding the AAC(3)-V activity was sequenced. The *aacC5* gene was localized in an open reading frame of 864 bp which encodes a polypeptide of *Mr* 31,070, which is consistent with the *Mr* (32,000) of the AAC(3)-V enzyme determined by physicochemical methods. High resolution S1 nuclease mapping suggests that transcription starts at or near the A residue of the ATG initiator codon. A DNA fragment from the 5′ region of *aacC5* had promoter activity in the promoter-probe plasmid pIJ486. The primary structure of the AAC(3)-V enzyme shows strong homology towards those of AAC(3)-III and AAC(3)-IV enzymes encoded by plasmids in gram-negative bacteriae genera. Upstream of the *aacC5* gene there is an open reading frame of 357 nucleotides (*orf357*) which does not appear to be involved in the expression of the *aacC5* gene.

121. Streptomycin-(3″)-phosphotransferase: a second resistance gene in streptomycin producer *S. griseus*

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A second Streptomycin (SM)-resistance gene has been localized and mapped on a 1·6 kb genomic DNA-fragment (pPHD 405) from *S. griseus* ATCC 10971 by cloning and subcloning in *S. lividans* TK 23. The gene, *aphE*, like the former described *aphD* gene, codes for a SM-phosphotransferase. DNA-segments necessary for double-strand sequencing according to Sanger were got by directed subcloning of restriction fragments and Bal31-shortening of the origin-clone in pUC-vectors. The sequencing of a 2,8 kb segment in the region of the *aphE* gene revealed two open reading frames with the length of 819 bp (*aphE*) and 894 bp (ORF) potentially coding for 29 kD and 31 kD proteins. Both genes are oppositely orientated and seem to share a common region for transcription termination. The *aphE* gene shows significant homologies, even in the 5′-upstream region, to the aph gene of Neomycin producer *S. fradiae*, coding for a Neomycin 3′-phosphotransferase.

The phosphorylated product of the *aphE* coded enzyme has been isolated from cell free extract

and showed the coupling pattern of a SM-3″-phosphotransferase when examined with 13C-NMR spectroscopy. This improved the APH(3″) enzyme as a gene product of *aphE* in contrast to *aphD*, coding for an APH(6) enzyme.

The substrate profile of the APH(3″) according to the aminoglycosides SM, Neomycin and Kanamycin confirmed the substrate recognition only for SM. The APH(3″) is also expressed in *E. coli*, probably under control of an *E. coli*-promotor. The similarities between the APH(3″) primary structure and the closely related APH(3′) enzymes primary structure and the closely related APH(3′) enzymes primary structure and also their relationship to other antibiotic- and protein-kinases is discussed with regard to a common origin.

122. Genetic instability and hypervariability correlated with DNA amplification in *Streptomyces ambofaciens*

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The wild type (WT) strain *Streptomyces ambofaciens* DSM 40697 exhibits a high degree of genetic instability. A frequency of about 1 per cent of pigment-defective colonies was observed in the progency of WT colonies. In contrast, auxotrophic mutants appeared at the frequency of 5×10^{-5}. Therefore, this genetic instability seems to preferentially affect characters involved in pigmentation and differentiation processes.

Out of 165 pigment-defective colonies independently isolated, only 21 per cent gave rise to an homogenous progeny exhibiting the parental phenotype. 87 per cent of the mutant colonies gave rise to an heterogenous progeny. Such a progeny was characterized by the absence of a preponderant phenotype and the number of phenotypes ranged from 2 to 8, most often 4 or 5. This new phenomenon of high mutability was called "hypervariability".

Molecular analysis of the total DNA of 71 independent clones arising in hypervariable progenies revealed highly amplified DNA in 21 per cent of the clones, while amplification was detected neither in 44 independent stable pigment-defective nor in 77 independent WT colonies. These results indicated that the hypervariability and amplification phenomena were correlated. The size of the amplified DNA sequences (ADS) ranged from 5 kb to 52 kb and the extent of the amplification could reach 50 per cent of the total genomic DNA. The restriction patterns analysis suggested overlaps between several ADS. This fact is in accordance with the notion of amplifiable regions in the WT genome.

123. Interspecific cloning of the sinefungin biosynthetic pathway from *S. incarnatus* into *S. lividans*

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Sinefungin is an antiparasitic and antifongic nucleoside antibiotic in which a molecule of ornithine is linked to the 5′ end of adenosine through a C-C bond.

Studies on sinefungin biosynthesis performed *in vivo* and *in vitro* showed that the direct precursor of the side chain of the antibiotic is arginine. Sinefungin may be produced directly from arginine and ATP by a cell free extract of *S. incarnatus*. However the yield is very low. In order to increase the yield of the enzyme responsible of the lost step of the biosynthesis involving the formation of the C-C bond we decided to clone the sinefungin biosynthetic genes.

We detected in *S. incarnatus* a plasmid carrying the genes coding for thiostrepton resistance but not for sinefungin production. The cloning by shot gun of the chromosomal DNA of *S. incarnatus*, partially digested by MboI enzyme, was carried out into the BamHI side of pIJ61 vector.*

The aph^{-} tsr^{R} colonies of *S. lividans* were tested for the production of antifongic and antiparasitic metabolites. Among 2300 colonies checked two produced an antibiotic active against fungi and parasites. The transformants were named *S. lividans* LI-4, *S. lividans* LI-1 respectively. HPLC analysis of the deproteinised fermentation culture of *S. lividans* LI-4 showed the presence of a metabolite with identical retention time to sinefungin. The antibiotic was isolated. Spectral analysis give the following characteristics: UV λ_{max} H_20 260 nm, ^{1}H NMR 400 MHz showed the presence of the 2H and 8H protons of adenine and also the protons of ribose and of side chain.

* This part of the work was performed at the Institut Pasteur à Paris, Department of Biotechnologie, Laboratoire de Génie Microbiologique, in collaboration with Dr Julian Davies.

The strain *S. lividans* LI-4 is growing slower than *S. lividans* pIJ61 with protoplastisation of the mycelium. The yield of the plasmid was about 10 fold lower than from *S. lividans* pIJ61.

A plasmid preparation from the transformant *S. lividans* LI-4 shows the presence of plasmids ranging between 40 Kb to 2 Kb. Transformation the strain *S. lividans* 66 by the plasmid mixture gave 30 per cent of active colonies. The BamHI digest of LI-4 was subcloned into the BamHI side of the plasmid pIJ61.

30 active transformants aph^{-} tsr^{R} were studied. The same instability of the plasmid was observed. The SDS-PAGE acrylamide electrophoresis showed some differences between the proteins of *S. lividans* 66, *S. Lividans* pIJ61 and the active transformants.

In order to avoid instability of the cloned insert in *S. lividans* the Hind III digest of LI-4 was cloned into the Hind III side of PUC-19 and used to transform *E. coli* TG1. The protein produced by the transformants carrying the insert of *S. incarnatus* were analysed by SDS-PAGE acrylamide gel electrophoresis.

The 15 Kb BamHI fragment of the insert from one of the transformant was isolated from agarose and subcloned into Bg1II site of the plasmid vector pIJ702. Transformants of *S. lividans* by the above plasmid were mel^{-} tsr^{R} and had antibiotic activity. Studies of sinefungin expression by fragments of the 15 Kb insert are now under way in the laboratory.

124. Structural instability of bifunctional plasmid pZG1 and single-stranded DNA formation in *Streptomyces*

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The possible mechanism of structural instability in *Streptomyces* of a hybrid plasmid pZG1 consisting of *Escherichia coli* pBR322 and *Streptomyces* pIJ350 plasmids has been studied. After transformation of *S. lividans* 1326 and *S. rimosus* R6 protoplasts with pZG1, transformants harboured the intact pZG1, pIJ350-like or variously deleted plasmid forms. However, the pattern of deleted plasmids in the cells varied in respect to the original transformant colony age and changed upon their subcultivation. The presence of intact pZG1 in at least a part of the *Streptomyces* colonies indicated the ability of pZG1 to replicate in the transformants and that deletion events occurred after at least one round of replication. Restriction analysis of deleted plasmids revealed the recombination and deletion events occurring close to the pBR322 replication origin. The interesting observation is that pZG1 is stabilized in *S. rimosus* R6 when this strain was transformed with pZG1 DNA isolated from the same strain, suggesting that upon repeated passage of pZG1 in *S. rimosus* R6 the plasmid DNA can become modified and thus protected from subsequent deletion, in analogy to the well described restriction-modification systems in eubacteria. The recombinant plasmid pZG1 and its variously deleted derivatives were found in *S. lividans* 1326 and *S. rimosus* R6 as double- and single-stranded DNA molecules. According to this, the phenomenon of pZG1 structural instability could be at least in part explained by rearrangements and the intracellular presence of single-stranded DNA as described for some high-copy broad host range *Bacillus subtilis* plasmids.

125. Cloning and comparative sequence analysis of the gene coding for isopenicillin N synthetase in streptomycetes

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The ability to produce β-lactam antibiotics is widespread among fungi such as *Penicillium*, *Acremonium* and *Aspergillus*, procaryotes such as *Streptomyces*, *Nocardia* and the genera of gram-negative bacteria including *Pseudomonas*, *Gluconobacter* and *Acetobacter*. In both the eucaryotes and procaryotes the early part of the biosynthetic pathway leading to the formation of penicillins and cephalosporins, is essentially the same. Isopenicillin N, the first β-lactam intermediate, is obtained by the cyclization of the linear cyclic tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-

valine (ACV) to form isopenicillin N. The enzyme catalyzing this reaction is known as isopenicillin N synthetase (IPNS). Successful cloning of a gene coding for IPNS was reported for three fungal strains and very recently for *Streptomyces clavuligerus*. In order to systematically explore comparative relations among the IPNS genes in a variety of β-lactam producers we have used the *S. clavuligerus* cloned gene as a reference probe. It is shown that the DNA of *S. jumonjinensis*, *S. lipmanii*, *S. cattleya*, *S. griseus*, and *S. lactamdurans* is able to hybridize with the probe constructed from the *S. clavuligerus* IPNS gene. Some other strains that were tested, including *S. lividans*, *S. venezuella* and *S. coelicolor* that are known to be unable to make β-lactam antibiotics, failed to respond with this probe. We report here the cloning of the IPNS genes of *S. lipmanii* and *S. jumonjinensis* from recombinant lambda genomic libraries. Comparative sequence analysis of *Streptomyces* IPNS genes and the reported fungal IPNS genes is presented. This type of study is being extended to other β-lactam producing microorganisms and is expected to reveal phylogenetic relationships between the β-lactam producers. It may also help to identify regions of the gene that have been conserved during evolution and are probably functionally important.

126. Site specific integration of pSAM2 plasmid in *Streptomyces*

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Streptomyces ambofaciens ATCC 23877 contains the 11·1 kb integrated plasmid pSAM2. This sequence is able, after excision and transfer, to integrate in *S. ambofaciens* DSM 40697 (devoid of any pSAM2 sequence) as well as in *S. lavidans* 66. The integration is site-specific and the recombination event occurs within a 49 bp sequence common to *S. lividans* 66 and *S. ambofaciens* DSM 40697 chromosomes and to the free copy of pSAM2. The two chromosomal integration zones cross-hybridize in Southern experiments. DNA sequence analysis reveals that homology at only the right side of the attachment site is responsible for hybridization. An open reading frame is present is conserved region and its possible involvement in pSAM2 integration is under study.

127. Male-programme in *Brassica*: cytological and biochemical kinetics

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In order to study male gemetophyte development in *Brassica oleracea* L., the various stages of microspores and pollen were assessed in fresh anthers with the use of fluorescent dyes and cytological observations. Developmental variations in SDS-PAGE protein patterns of anthers and pollen grains were then analysed from the late vacuolate microspore stage to the end of the pollen maturation period. Total protein staining and concanavalin A-binding glycoprotein detection showed that a specific set of developmental polypeptides appears during the tricellular pollen stages. Protein synthesis was studied by [^{35}S]-methionine incorporation, SDS-PAGE, and autoradiography. Two periods in the protein synthetic activity were detected: the first one corresponding to the microspore and bicellular pollen stages; the second one corresponding to the mid, late, and mature tricellular pollen stages. During this second period, new polypeptides are synthetized and most of them are correlated with the developmental polypeptides that appear in total and Con A-binding protein patterns. These results suggest the occurrence of a metabolic reorientation after the second pollen mitosis, at the time of sperm cell maturation. The biochemical data are discussed in terms of diploid and haploid genome expression.

In order to elucidate the genetic control of the sperm cell formation we have developed a technique to isolate the germ cells. Like that we have a potential tool to raise monoclonal antibodies against the sperm cells for further molecular genetic control.

128. Use of monoclonal antibodies to determine putative S-products of pollen in *Brassica oleracea*

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S-locus specific glycoproteins (SLSGs) were purified by electrophoretical procedures from stigmas of a *Brassica oleracea* L. genotype homozygous for the S6 allele of self-incompatibility. Several monoclonal antibodies (MAbs) were raised against the S6 specific glycoproteins. Among them, MAb 97–53 was proved to be likely directed against the polypeptide core of the S6 glycoproteins and allowed to detect about 15 antigenic compounds in the S6-stigma extracts. Besides, antigenic homologies were found between SLSGs of four different genotypes of *Brassica oleracea* L., homozygous respectively for the S6, S13, S14 and S22 alleles.

On the pollen side, proteins binding to MAb 97–53 were characterized on electroblots. These compounds constitute putative candidates for the S-specific molecules of the male gametophyte. An interesting point of our study is that no cross-reaction between pollen and stigma proteins of similar molecular mass was observed. This suggests that S-specific substances in pollen may have different molecular structures from S-glycoproteins in the stigma. Lastly, even though we cannot say for certain that the cross-reacting material in pollen is associated with the S gene expression, the MAb technology appears a promising technique in the study of the self-incompatibility at the molecular level.

129. Late-acting self-incompatibility in *Tabebuia caraiba* and *T. ochracea* (Bignoniaceae)

Tabebuia caraiba (Mart.) Bur. and *T. ochracea* (Cham.) Standl. are two common species of the cerrado or savanna-like vegetation which extends across central Brazil. Both congeners occur sympatrically and individuals of each species may grow within a few metres of each other.

Studies on the floral phenology of these species have shown that although *T. caraiba* initiates flowering first there is an overlap in flowering period. Furthermore, both species share the same spectrum of large- to medium sized bees as pollinating vectors.

Controlled pollinations indicate that both *T. caraiba* and *T. ochracea* are strongly self-incompatible and inter-incompatible although fluorescence microscopy has revealed that in both species self-pollen tubes grow to the ovary and penetrate ovules. Both species, therefore, have "late-acting self-incompatibility" (sensu Seavey and Bawa, *Botanical Review 52*, 195–219, 1986). In reciprocal inter-specific pollinations pollen tubes also grow to the ovary and can be seen to penetrate some ovules suggesting that inter-specific incompatibility in this case is also of a late-acting type. Cytological events subsequent to ovule penetration by self- or inter-specific pollen tubes remain to be elucidated.

130. A chromosomal basis for change in breeding system in a complex interchange heterozygote?

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In plants, the genetic system of complex interchange heterozygosity leading to permanent hybridity is usually accompanied by a change in breeding system. This phenomenon has attracted the attention of numerous biologists, but the mechanism for the change has largely remained elusive.

Gibasis pulchella is a monocot (family Commelinaceae) that exhibits gametophytic incompatibility. Diploid populations ($2n = 10$) contain a mixture of structural homozygotes and/or single-interchange heterozygotes (both self-incompatible) and complex interchange heterozygotes (self-compatible). This clear-cut difference in breeding system and the very large, easily identifiable chromosomes of *G. pulchella* has encouraged a detailed cytogenetical investigation of the system.

Although complex-heterozygotes have been assembled via different chromosomal rearrangements, the 2,5-translocation, which transfers two nucleolus-organizing regions (NOR's) to the same chromosome, is almost exclusively present. Both of the NOR's on the new chromosome are active as shown by silver staining, an unexpected situation that has prompted speculation as to a possible function. Only one complex-heterozygote does not contain the 2,5-translocation. This genotype shows some unusual features, including inability to achieve self-fertilization. The 2,5-translocation may be significant in the change in breeding system from self-incompatible to self-compatible.