MOLECULAR GENETICS OF BACTERIAL PENICILLINASE

Analysis by genetic transformation of penicillinase mutants in Bacillus licheniformis

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The genetic transformation system in *Bacillus licheniformis* strain 9945A (Thorne and Stull, 1966) has allowed both nutritional markers and penicillinase mutations to be transformed at high frequencies. Large numbers of penicillinase transformants were obtained for analysis by selecting *ilvD1*, a marker weakly linked to the penicillinase genes (*pen*).

A phased appearance of *ade-1* and *ilvD1/pen* transformants was obtained when transforming DNA was added at different times to a competent recipient culture (Sa/1; *ade-1 ilvD1 pen i+p+*) in transformation medium (TM). The linkage of *ilvD1* to *pen* was variable in such experiments, being highest when numbers of *ilvD1* transformants were greatest. The results indicate that competent cells are synchronized in TM and it is proposed that integration of DNA occurs at a specific position on the replicating genome at any given time.

*Ile-1*, *mat-1* and an 'aromatic' cluster of genes were all linked to *ilvD1*, confirming the similarity of the *Bacillus subtilis* and *B. licheniformis* genomes and indicating that *pen* is a terminally replicated marker in *B. licheniformis*.

A suppressor mutation (*str-1*), which is unlinked to the penicillinase genes, and apparent nonsense mutations in the penicillinase structural gene (*p*) have been characterized.

A genetic analysis of penicillinase regulatory gene mutations indicates that the synthesis of penicillinase is controlled negatively by the product (*R*) of gene *i* which is closely linked to *p*. Mutations in *i* can give either a magno-constitutive phenotype (fully induced level, uninducible) or a basal uninducible level. Other regulatory gene mutations, giving a basal level of penicillinase and defective inducibility, map in at least two other genes. Preliminary
protein structural evidence and genetic results suggest that the
penicillinase operator (0) maps on the i-distal side of p.

A model for penicillinase regulation, based on the results of
this thesis and on biochemical studies of penicillinase induction, is
presented. The applicability of this model to other control systems
in which the cell has a permeability barrier to external substrate
and effector is discussed.
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INTRODUCTION
1. GENERAL INTRODUCTION

Observations that bacteria can change their enzymic composition when exposed to different chemical environments have been reported since the end of the last century (review: Pollock, 1959). Such adaption to environmental change may be genotypic, resulting from mutation of the genetic material, or phenotypic, where the enzymic changes are due to an altered synthesis of particular enzymes in response to specific components of the environment. Monod and Cohn (1952) proposed that phenotypic adaption be described as induced synthesis of enzymes, the specific substance effecting the change being termed the inducer. A large number of inducible systems have been discovered and studied in bacteria, though most of the fundamental characteristics of the induction process were established in the study of the lactose (lac) system of Escherichia coli. Induction of one of the lac enzymes, β-galactosidase, which hydrolyses lactose and other β-galactosides, was shown to involve the appearance of a new antigen (Cohn and Torriani, 1952). Subsequently the de novo synthesis of β-galactosidase on induction was demonstrated (Hogness et al., 1955).

Benzer (1953) showed that in a bacterial population, gratuitous induction (i.e. induction under conditions in which inducer is neither metabolized nor has any other metabolic effect) of β-galactosidase proceeded uniformly in all cells. However at low inducer concentrations, or where inducer was the sole carbon source, the response to induction was heterogeneous. Induction of β-galactosidase is complicated by the need for an inducible permease to transport inducer into the cell. Therefore detailed studies of the induction kinetics of β-galactosidase were made in permeaseless mutants of E. coli (Boesi and Cowie, 1961).

Using the gratuitous inducer isopropyl-thio-β-D-galactoside (IPTG) at a saturating concentration, a lag of 5 min. at 37°C, was observed,
before the maximum differential rate of $\beta$-galactosidase synthesis was attained. On the removal of inducer there was a lag of 2-3 min. before differential enzyme synthesis decreased. These observations led to the conclusions that the enzyme-forming unit is unstable and must be synthesized continuously to maintain specific enzyme synthesis (Boezie and Cowie, 1961; Roberts et al., 1961).

As the basic outlines of the steps involved in the transfer of information from genes to proteins were elucidated, it became practicable to consider regulation of specific protein synthesis in terms of the properties of the protein biosynthetic mechanisms. A genetic study of *E. coli* mutants altered in their control of enzyme synthesis in the *lac* system, led to the proposal by Jacob and Monod (1961) of a system of negative transcriptional control for the regulation of specific enzyme synthesis. Considerable genetic and biochemical data supported their hypothesis and in 1965 the isolation of their postulated repressor and its subsequent biochemical characterization (Gilbert and Müller-Hill, 1966; Riggs and Bourgeois, 1968 and 1969; Riggs et al., 1968), confirmed their hypothesis in almost every respect.

Many other regulatory systems are being studied in bacteria and viruses. As well as inducible systems there are repressible systems, often biosynthetic pathways, in which a specific effector represses enzyme synthesis.

Results from some inducible and repressible systems can readily be interpreted as indicating negative control by an allosteric repressor, while others are difficult to reconcile with this type of regulation in its present form. Whether these differences reflect a basic difference in type of control or whether they can be accounted for by a modified repressor-operator-operon model of control will
become more apparent with time. A difficulty in studying many of the systems is the unavailability of the desirable genetic techniques and the lack of a suitable means of studying protein gene-products.

Despite such difficulties a number of systems have been analysed in detail and the results suggest that rather than negative transcriptional control, some systems are subject to positive control, a control gene product being required for the expression of genes under its control (Sheppard and Englesberg, 1967; Power, 1967).

Like $\beta$-galactosidase, penicillinase (E.C.3.5.2.6.) is an inducible enzyme in a number of bacterial species. That the induction is due to a de novo synthesis of protein has been shown for Bacillus cereus (Pollock and Kramer, 1958). Penicillinase hydrolyses the $\beta$-lactam bond of penicillins and cephalosporins (appendix IX), conferring resistance to these antibiotics, in bacteria producing the enzyme. The physiology of induction of penicillinase in B.cereus, Bacillus licheniformis, and Staphylococcus aureus has been extensively studied. Induction in these organisms is markedly different from the induction of $\beta$-galactosidase in E.coli; there is no permeability barrier between substrate and penicillinase and a transient contact with inducer is sufficient to induce enzyme formation. After contact with inducer, the maximal differential rate of enzyme synthesis is not attained for at least 20 min., and on the removal of inducer, enzyme synthesis continues for about 2 hours before being repressed (Pollock, 1952; Davies, 1969; Imsande, 1969).

The enzymological and serological properties of the penicillinas have been well characterized (review: Citri and Pollock, 1966). All the penicillinas investigated are single polypeptide chains of relatively low molecular weight, making them amenable to investigation by protein chemical techniques. Many penicillinas mutations have been
isolated in strains of *E. coli*, *E. licheniformis* and *E. aerogenes*, and a large number of these mutants have been characterized biochemically and physiologically. Therefore with the vast amount of biochemical and physiological data available, it is of great interest to investigate penicillinase regulation at the genetic level, to correlate the genetic results with the biochemical data, and to compare penicillinase regulation in different organisms to regulation in the *lac* and other well characterized systems.

2. REGULATION OF INDUCED ENZYME SYNTHESIS

a) The *lac* system in *E. coli*

The *lac* system consists of three closely linked structural genes (Jacob and Monod, 1961; Jacob et al., 1965). The *z* gene gives rise to the enzyme β-galactosidase which hydrolyses β-galactosides. Adjacent to the *z* gene is the *x* gene which codes for a permease, responsible for transporting galactosides into the cell. The third gene *y*, next to *x*, codes for the enzyme thiogalactoside transacetylase, whose physiological function is unknown. The level of all three enzymes is co-ordinately controlled on induction and repression. This observation led to the suggestion that the three genes are controlled as a single unit, termed an operon (Jacob and Monod, 1961). Jacob and Monod proposed that at one end of the operon is a 'switch', a site called the operator, while outside the operon is another gene (*i*-gene), which synthesises a diffusible product, the repressor. The repressor binds specifically with the operator in an uninduced cell, so as to block expression of the operon; therefore the control in this system is negative. On induction, inducer binds with repressor, displacing it from the operator site and enzyme synthesis can begin.

The evidence for this model was mainly obtained from a study of
mutants in which the regulation system had broken down. The first mutants studied were termed constitutive (i−) (Cohen-Bazire and Jolit, 1953) and they synthesized greater than fully induced levels of β-galactosidase, even in the absence of inducer. These constitutive mutants synthesized enzyme indistinguishable from that produced in uninduced cells (Rickenberg et al., 1956) and mapped together in a region linked to the structural genes (Jacob and Monod, 1961). E.coli is normally haploid, but stable diploids can be constructed either by "sexduction" (Jacob and Adelberg, 1959), using F factors, or by using temperate bacteriophages carrying the lac region (e.g. φ80d, Beckwith and Signer, 1966). In diploids carrying an i− mutation together with an intact i− gene (i+), both operons were found to be fully inducible. Therefore an intact i+ can exert its effect on an operon carried by another chromosome (trans-dominance). Another type of constitutive mutation is predicted by the operon concept. This will be a mutation in the operator region, reducing or abolishing its affinity for repressor. Such operator constitutive mutations (o+) should be cis-dominant in the presence of a second intact lac region, since the repressor produced by the i− gene in the transposition will not be able to exert its effect on the mutated operator. Mutants with these properties were isolated by mutagenesis of i+/i+ diploids; thus virtually eliminating recessive i− mutations (Jacob et al., 1960).

From recombination data, o+ mutants were found to map between the i and z region (Jacob, Ullman and Monod, 1964). Most o+ mutants cannot be reverted and are therefore thought to be deletions or multi-site mutations. In the absence of inducer, o+ mutants synthesize 10-20% of the amount of enzyme produced by an i− mutant. On induction they synthesize the fully induced levels of enzyme.

Another class of regulatory gene mutations which mapped within
the i-gene were later found (Wilson et al., 1964). These mutations gave rise to a \textit{lac} phenotype and produced basal amounts of the structural gene enzymes in the presence or absence of inducer. When such mutants were combined with an \textit{i}\textsuperscript{+} or \textit{i}\textsuperscript{−} gene in diploids, the uninduced level of structural gene expression was observed in the presence or absence of inducer. These mutations were termed \textit{i}\textsuperscript{S} (super-repressor), and their repressors were assumed to have lost their affinity for binding inducer. Treatment of \textit{i}\textsuperscript{S} mutations with mutagens gives rise to constitutives of \textit{i}\textsuperscript{−}-type and \textit{o}\textsuperscript{0}-type at high frequency.

Besides a repressor binding site, an operon requires sites for the binding of RNA polymerase and for the initiation of transcription, though these may be the same. Mutation at such sites might be expected to give rise to a reduced rate of expression of the operon. All mutations characterized as \textit{o}\textsuperscript{0} allow maximal expression of the operon even though these mutations are deletions or multi-site mutations (Jacob et al., 1964). Therefore \textit{o}\textsuperscript{0} mutations do not include initiation mutations. Deletions covering \textit{o} and extending into \textit{z} were isolated (Jacob et al., 1964); none of these gave rise to operon function (measured by the production of \textit{y} protein). These observations led to the proposal of a site, the promoter, required for operon expression and mapping between \textit{o} and \textit{z}. A number of mutants were later isolated which resulted in a lowered expression of the \textit{lac} operon (Scaife and Beckwith, 1966), though near normal inducibility was retained. These mutants were termed promoter mutants and were originally thought to map between \textit{o} and \textit{z}, in the promoter region of Jacob et al. However a subsequent more conclusive genetic analysis showed these mutants to lie between \textit{o} and \textit{i} (Ippen et al., 1966; Miller et al., 1968). The term promoter mutant has been retained for these mutants and the promoter region is now defined as lying between \textit{i} and \textit{o}. Promoter
mutants have been shown to be cis-dominant in heterozygotes (Scaife and Beckwith, 1966; Ippen et al., 1968) and some of those isolated are transition point mutations, as determined by reversion studies (Arditti et al., 1968).

The final proof of negative control by an allosteric repressor in the lac system was the isolation of the lac repressor and its subsequent characterization (Gilbert and Miller-Hill, 1966 and 1967; Riggs and Bourgeois, 1968 and 1969; Riggs et al., 1968). The repressor is a protein tetramer of M.W. about 150,000. It only binds to native bi-helical DNA containing the lac operator region. The binding disappears in the presence of the inducer IPTG and is increased in the presence of the anti-inducer o-nitrophenyl β-D-fucoside. Binding of lac repressor to DNA carrying a lac o° mutation is diminished in proportion to the constitutivity conferred by the o° mutation. Strains carrying deletions or nonsense mutations in the i-gene produce no isolatable repressor and strains temperature-sensitive for lac repression give rise to a temperature sensitive protein repressor.

The kinetics of the interaction of the repressor with operator have been studied (Riggs and Bourgeois, 1969) and can be explained by the equilibrium:

\[
\text{repressor} + \text{operator} \rightleftharpoons \text{repressor-operator complex}
\]

The dissociation constant for repressor-operator complex is \(2 \times 10^{-12}\)M. and the rate constants are: \(k_f = 10^8 M^{-1} \text{sec}^{-1}\), \(k_b = 4 \times 10^{-4} \text{sec}^{-1}\).

The specific binding of repressor to operator is being used for the purification of the lac operator region (Riggs and Bourgeois, 1969) and it should therefore be feasible to determine the nucleotide sequence of this region of the DNA.
Similar studies using the specific binding of RNA polymerase to DNA should enable a determination of the structure of the promoter region of the DNA. It is not yet known whether the operator region is transcribed, nor is the point of initiation of translation known, though the answers to these problems should soon be provided with the considerable effort that is being put into understanding the regulation of the lac system in terms of the structures of the molecules involved.

b) Other systems

The operon model of negative control provides an explanation of the behaviour of a number of other regulatory systems in bacteria.

Three linked structural genes synthesize the enzymes responsible for the fermentation of galactose in E. coli. These genes are controlled co-ordinately by an unlinked regulatory gene (Buttin, 1963).

The repression of enzyme synthesis in biosynthetic pathways can be explained by the negative control of a repressor. In this case the active repressor consists of a complex between control gene product and specific effector, which usually is either the end-product of the biosynthetic pathway, or a derivative of it. The two classes of constitutive in these repressible systems are analogous to those in the lac system: mutations in the regulatory gene coding for repressor are compensated by an intact copy of the repressor gene in diploids (Roth et al., 1966; Ito and Crawford, 1965; Maas and Clark, 1964). Constitutives that are expressed in the heterozygous diploid state define the operator (Hiraga, 1969; Ramakrishnan and Adelberg, 1964 and 1965).

A number of regulatory systems cannot be fitted into this scheme of negative control. One such system is the arabinose system of E. coli, which is inducible and synthesizes the enzymes responsible for utilizing L-arabinose (Sheppard and Englesberg, 1967). Three
structural genes (B, A and D) are closely linked and are controlled co-ordinately by a linked control gene (C), which can exist in at least three allelic forms.

1. $C^+$ (wild-type), inducible for the enzymes specified by the structural genes.

2. $C^-$, all three enzymes uninducible and unexpressed.

3. $C^0$, all three enzymes expressed constitutively.

Some $C^0$ mutations have been shown to map between $C^-$ mutant sites. Dominance relationships in diploids have shown that both $C^-$ and $C^0$ mutations are compensated by a wild-type $C^+$ gene to give a fully inducible phenotype. In a $C^-/C^0$ diploid the structural genes are expressed constitutively. Deletion mutants that are probably confined to the C gene give rise to a $C^-$ phenotype, whilst a second class of deletions give rise to a pleiotropic negative phenotype and provide evidence for the operon 'switch' or initiator region (I). It therefore seems that for expression in this system production of C-gene product is required as well as an intact initiator region (I).

The following model has been proposed: C produces a gene product $P_1$ that is converted to $P_2$ in the presence of inducer. $P_2$ binds the initiator region and expression of the operon can begin. $C^-$ mutants make an altered $P_1$ that can no longer bind inducer and the operon remains unexpressed. $C^0$ mutants give rise to a product that can bind I and therefore give rise to continuous expression of the operon (Sheppard and Englesberg, 1967). If this hypothesis is true one has to explain the fact that a $C^+/C^0$ diploid is fully inducible. One explanation is that the C gene product is polymeric and a mixed polymer of wild-type and mutant monomers does not bind to I in the absence of inducer. A second explanation is that $P_1$ in the absence of inducer actively represses expression of the operon and that this
repression overrides the positive effect of the $o^\circ$ gene product on $I$. It now seems that this second explanation is the correct one. Studies with deletion mutants (Englesberg and Squires, 1968) suggest a genetically distinct operator region which is subject to repression by $P_1$.

![Diagram](image)

It is becoming increasingly evident that a large number of bacterial and phage systems are regulated in part by a positive control system. The enzymes of the rhamnose utilizing pathway seem to be subject to positive control (Power, 1967) as do the maltose utilizing enzymes of *E. coli* (Hatfield *et al.*, 1969). A large number of bacteriophage and T4 functions also seem subject to positive control (Thomas, 1966 and 1968; Snyder and Geiduschek, 1968).

The situation with alkaline phosphatase of *E. coli* is still very confused. Very little enzyme is synthesized in the presence of excess orthophosphate; derepression occurs in the presence of low phosphate, (Echols *et al.*, 1961). Genetic derepression arises by mutation in either of two regulatory genes, R1 and R2. R1 is closely linked to the structural gene for alkaline phosphatase while R2 is unlinked. Constitutive mutations in either R1 or R2 are recessive to the wild-type alleles in diploids. Other mutations in R1 give rise to a lowered constitutive expression of alkaline phosphatase; this level cannot be elevated by derepression. This effect is recessive in diploids and can be eliminated by combining this R1 mutation with an R2 mutant (Garen and Echols, 1962a).
In addition there are R1 mutants that lack alkaline phosphatase and are also recessive (Garen and Echols, 1962b). The gene product of the R2 gene has been isolated (Garen and Otsuji, 1964), and shown to be concomitantly repressed with the alkaline phosphatase. Some R2 constitutive mutants, which were shown to be nonsense mutations still synthesized R2 protein and therefore the R2 region was divided into two cistrons; R2a, specifying the isolated protein, and R2b. Moreover the synthesis of the R2a-coded protein was not affected in a strain carrying a R1 mutation which gave rise to the underepressible level of alkaline phosphatase.

The interpretation of these results is difficult and as yet no satisfactory model has been put forward for alkaline phosphatase regulation in *E. coli*.

From the examples described above, it can be seen that for a genetic study of regulation an effective means of genetic transfer and analysis is required. It is also desirable to have a means of constructing heterozygotes so that the dominance relationships between allelic genes and their products can be studied. For a complete understanding of a system genetic results must be correlated with biochemical studies of the components of the system. To enable such studies to be made, the techniques for isolating and characterizing the protein and nucleic acid components of the system are required.

c) *Staphylococcal penicillinase*


In a large number of staphylococcal strains, the penicillinase
genes are carried on an extrachromosomal plasmid (Novick, 1963), along with a number of other genetic determinants. These include resistance to a variety of metal ions (Moore, 1960; Richmond and John, 1964; Novick, 1967) and resistance to macrolide antibiotics such as erythromycin (Hashimoto, Kono and Mitsuhashi, 1964). The penicillinase genes are usually transduced along with the whole plasmid into the new host (Novick and Richmond, 1965). Certain combinations of different plasmids can co-exist in the same cell, giving relatively stable diploids, while other combinations are unstable and segregate rapidly, giving recombinants.

The results of an analysis of regulatory-gene mutants in the staphylococcal penicillinase system, suggest that regulation is analogous to that in the lac system. Constitutive mutations are recessive in diploids to an intact i-gene (i⁺) (Richmond, 1965). However, no c⁰-type mutants have been isolated despite an extensive search by mutagenization of i⁺/i⁺ diploids (Smith and Richmond, unpublished).

Recently a strain of S. aureus with a chromosomal location for its penicillinase genes has been characterized (Asheshov, 1966). This strain has allowed a study of penicillinase synthesis in diploids between the chromosomal and plasmid penicillinase genes (Asheshov and Dyke, 1968). These experiments have shown that, unlike with plasmid/plasmid diploids, the constitutive synthesis of penicillinase by a plasmid-borne i⁻ mutation is not completely repressed in the presence of a chromosomal i⁺ gene. However in the reciprocal situation the constitutive synthesis of penicillinase by the chromosomal genes is completely repressed in the presence of a i⁺ gene carried by a plasmid.

A number of other types of regulatory-type mutation have been isolated and studied in S. aureus. Mutagenesis of a wild-type inducible
strain of *S. aureus* gave rise to a class of mutants producing basal level of enzyme; induction tests showed these mutants to be non- or semi-inducible, (Richmond, 1967). In the presence of a second copy of the penicillinase genes, producing a constitutive level of penicillinase (i−p+), such non-inducible strains gave rise to the fully inducible level of enzyme, 50% being derived from each of the structural genes. Other mutants, derived from a wild-type inducible strain, synthesized very low levels of enzyme and were either inducible (micro-inducible), or uninducible (micro-constitutive) (Richmond, 1966). These mutations only affected the expression of the structural genes co-linear (cis) to the mutation in diploids, while a gene product synthesized by the plasmid carrying these mutations was able to restore inducibility to a plasmid synthesizing penicillinase constitutively.

A chromosomal mutation of *S. aureus*, resulting in constitutive synthesis of penicillinase has been reported (Cohen and Sweeney, 1968). This mutation (Rf−) has the ability to confer constitutive penicillinase synthesis to plasmid-borne inducible penicillinase genes. The level of penicillinase synthesised in R2 containing strains at 32°C, was about the same as that synthesized by a plasmid penicillinase constitutive mutation (i−), though at 40°C, R2− mutants synthesized a reduced amount of enzyme; i− mutations normally give rise to full constitutive synthesis of penicillinase at both 32°C and 40°C.

One of the difficulties in analysing the regulatory-type penicillinase mutations carried by staphylococcal plasmids has been the difficulty in analysing recombination data (Smith, 1969). Recombination in this system is relatively infrequent and 3-factor reciprocal crosses have led to ambiguous results. However a deletion
analysis of penicillinase plasmids has been possible (Novick, 1967) and extensions of this analysis should allow the penicillinase mutations to be mapped accurately.

3. BACILLUS LICHENIFORMIS PENICILLINASE

A transformation system in B. licheniformis strains 749 and 6346 was used by Dubnau and Pollock (1965) to initiate a study of penicillinase regulation in this organism. The penicillinases synthesized by strains 749 and 6346 are distinguishable by their substrate profile and by their reaction with specific antisera (Pollock, 1965). Both enzymes are single polypeptide chains of molecular weight about 30,000; there are probably no more than three amino acid differences between the two proteins (Ambler, personal communication). The amino acid sequence of the 749-type enzyme has been determined (Meadway, 1969). Genetic hybrids between 749 and 6346 have been constructed by transformation (Dubnau and Pollock, 1965). Protein structural studies with the hybrid penicillinases synthesized by these hybrids are in progress; (Ambler et al., unpublished); from these studies it is hoped to locate the sites responsible for the differences in activity between the two penicillinases. It has also been shown that there is about 40% homology in sequence between B. licheniformis 749-type enzyme and a staphylococcal plasmid-coded penicillinase (Ambler and Meadway, 1969).

The synthesis of 749- and 6346-type penicillinases is inducible and 30-50% of the enzyme synthesized is released into the culture medium as exo-enzyme. The remaining cell-bound enzyme is still accessible to substrate and about 50% is accessible to specific antiserum (Kushner and Pollock, 1961). The cell-bound penicillinase can be released either by treatment with trypsin in hypertonic sucrose
(Kushner and Pollock, 1961), or by protoplasting cells under conditions in which they do not significantly lyse (Sargent et al., 1969a and b). The penicillinase released on protoplasting has been reported to be associated with large molecular weight membranous material (Sargent et al., 1969a and b). Recent electron microscopic studies (Ghosh et al., 1969) have indicated that the synthesis of penicillinase in \textit{B.licheniformis} strains 749 and its derivatives, is associated with vesicular structures occurring in invaginations of the cell-membrane. These structures were present in induced cells and in a constitutive strain, but absent in uninduced cells. However electron microscopic studies in this laboratory (Highton, 1969 and unpublished results), that were intended to show the cellular location of penicillinase in strains 749 and derivatives, did not confirm these results. Highton has shown that the fine-structure of mesosomes, as membranous structures lying in invaginations of the cell-membrane have been called (Fits-James, 1960), is very sensitive to the preparational conditions used. "Vesicles" observed after some treatments prior to fixation were shown to be produced by breakdown of mesosomal membranes (Highton, 1969). Only one mesosome per cell was seen in sections of cells, inducible, constitutive, or negative for penicillinase, under optimal conditions for the preparation of samples.

The release of cell-bound penicillinase has been studied in the absence of protein synthesis (Kushner and Pollock, 1961; Sargent et al., 1969a). The release is strongly pH and temperature dependent, and is inhibited by quinacrine and related compounds (aromatic, chlorinated tertiary amines). Such compounds have a variety of metabolic effects, affecting especially the properties of lipid-containing compounds (Sargent et al., 1969a). Mutants of \textit{B.licheniformis} altered in their ability to excrete penicillinase have
not been isolated.

The induction of \textit{B.licheniformis} penicillinase, like that of other penicillinases synthesized by gram-positive bacteria, is markedly different to the induction of \( \beta \)-galactosidase in \textit{E.coli}. With \( \beta \)-galactosidase induction, using IPTG as inducer, the maximal differential rate of enzyme synthesis is reached after about 5 min. at 37°C. (Boezi and Cowie, 1961). On the removal of inducer, repression of enzyme synthesis is equally rapid. These kinetics can be readily explained by the short half-life of \( \beta \) mRNA and a negative system of transcriptional control. After the addition of inducer to an exponentially growing culture of \textit{B.licheniformis}, the maximal differential rate of enzyme synthesis is not attained until one doubling time after induction is commenced (about 60 min.), (Davies, 1969). However a gradual differential increase in penicillinase synthesis can be measured 12 min. after induction. Similar results have been obtained for penicillinase induction in \textit{B.cereus} (Imsande, 1969). The maximal differential rate of enzyme synthesis is not observed for about a doubling time after the addition of inducer (about 45 min.), though an increased differential rate of synthesis of penicillinase can be measured after 30 sec. On removal of unbound inducer, repression of enzyme synthesis is observed only after about three generations (Pollock, 1958).

Such results have been explained in the past by a relatively stable penicillinase mRNA (Pollock, 1963; Yudkin, 1966). However, the results of recent experiments in which penicillinase synthesis has been studied after rifampicin and actinomycin D treatment of cells, suggest that the half-life of penicillinase mRNA in \textit{B.licheniformis} is 4.7 min. at 37°C. (Davies, 1969). Similar studies with \textit{B.cereus} give a half-life of about 2 min. (Davies and Imsande, unpublished).
The binding of penicillin to *B. licheniformis* is specific and follows saturation kinetics (Davies, unpublished results), similar to those for penicillin binding in *B. cereus* (Duerksen, 1964) and *S. aureus* (Rogers, 1967). The majority of this bound penicillin is insensitive to penicillinase and for *B. cereus* has been shown to be associated with lipo-protein (Duerksen, 1964). Protoplasting of cells which have bound penicillin, results in up to 95% loss of bound penicillin into the supernatant as a penicillin-lipoprotein complex. It seems quite likely that the penicillin binding sites for antibacterial action and for penicillinase induction are different (Rogers, 1967). A transient binding of penicillin is sufficient to give a maximal differential rate of penicillinase synthesis a doubling time later, and significant amounts of penicillin cannot be found associated with specific organelles within the cell (Duerksen, 1964). One is therefore faced with the topological problem of how the inducer (penicillin) which remains associated with some rigid structure outside the cell is able to affect specific mRNA synthesis via a control element, if the mechanism of regulation is transcriptional in the penicillinase system.

That the induction of penicillinase in cultures of *B. licheniformis* consists of a relatively homogeneous response of the whole population to inducer has been shown by a measurement of the distribution of penicillinase in single cells of *B. licheniformis* (Collins, 1964). Uninduced cells were shown to produce on average clusters of 1100 molecules of penicillinase (0.85 clusters/cell on average), while a constitutive population produced penicillinase in groups of 55000 molecules (0-7 per bacillus). It was deduced that enzyme partition can be highly asymmetric when a cell divides; suggesting that penicillinase molecules are associated with a structurally rigid
A large number of penicillinase mutants have been isolated in *B. licheniformis* strain 749 (Dubnau and Pollock, 1965). These mutants were classified as structural or regulatory on the basis of their enzymological and serological properties. Using a transformation system, Dubnau and Pollock (1965) showed a regulatory locus controlling inducibility to be linked to the penicillinase structural gene. However, a detailed analysis was not possible because of the poor transformation frequencies obtained \((10^{-5})\) and the difficulty in selecting penicillinase transformants. Subsequently Thorne and Stull (1966), investigating *B. licheniformis* transformation, reported high transformation frequencies using a colonial variant of strain 9945A. In this laboratory it was shown that strain 9945A produces a penicillinase indistinguishable from that synthesized by strain 749, judged by enzymological and serological tests (Fleming and Hill, unpublished). It was possible to transfer penicillinase mutations from strain 749 to strain 9945A, with the appearance of the original phenotype in the new host (Collins, unpublished).

A 10000 fold range of penicillinase activities can be distinguished by a test on agar plates containing up to 1000 bacterial colonies (Collins, unpublished). It is therefore possible to analyse large numbers of penicillinase transformants producing different levels of the enzyme. A similar range of penicillinase activities can be measured accurately in liquid culture.

For the work described in this thesis, it was therefore decided to use the transformation system in strain 9945A for the analysis of the many penicillinase mutants isolated in strain 749. There are three main limitations of the system. First a means of obtaining large numbers of penicillinase transformants is required. In the absence of
a practicable selection technique, a linked marker to the penicillinase genes is required, which when selected will give large numbers of penicillinase transformants. It was hoped to overcome this problem by finding a marker closely linked to penicillinase.

The second limitation is that transformation does not offer a means of studying the dominance relationships between allelic genes and their products in heterozygotes. Lack of complementation studies, restricts the interpretations that can be made as to the functional relationships between genes and their products. The one report of abortive transformation in *B. subtilis* which might have allowed such studies (Iyer, 1965) has recently been refuted (Jensen, 1969).

The third limitation was that at the beginning of this work very little information was available concerning the biochemistry and genetics of *B. licheniformis*. However, recently *B. licheniformis* has become the subject of much study. The biochemistry of sporulation (Spizizen, unpublished) and the nature and regulation of biochemical pathways are being studied (Nassar *et al.*, 1969; Gray and Bernlohr, 1969; Laishley and Bernlohr, 1969). Comparative studies with other bacilli have shown a close relationship to *B. subtilis*. Hybridization studies have shown a high degree of homology of ribosomal genes between species of bacilli (Dubnau *et al.*, 1965) and streptomycin resistance markers can be transformed between *B. subtilis* and *B. licheniformis* at a low frequency (Dubnau *et al.*, 1965; Goldberg *et al.*, 1966). A partial replication map of *B. licheniformis*, obtained by marker frequency analysis (Tyeryar *et al.*, 1966), shows a great similarity to the *B. subtilis* genetic map (Dubnau *et al.*, 1968). A number of *B. subtilis* transducing phages have been shown to transduce *B. licheniformis* effectively (Taylor and Thorne, 1963; Tyeryar *et al.*, 1968 and unpublished). It therefore seems that genetic techniques will
become available to allow a more detailed analysis to extend the experimental studies to be presented here.
MATERIALS & METHODS
1. MATERIALS

Benzylpenicillin, cephalosporin C, 7-aminocephalosporanic acid and streptomycin sulphate were gifts from Glaxo Ltd.

D-Cycloserine was obtained from the Sigma Chemical Co. Ltd (London).
O-Carbamyl-D-serine was a gift from the Commercial Solvent Corp. (N.Y.).
Aminopterin was bought from Koch-Light Laboratories Ltd.

Deoxyribonuclease 1. (DNA'ase), was obtained from either the Worthington Biochemical Corporation or the Sigma Chemical Co. Ltd (London).
Polyvinyl alcohol (0-600) was obtained from Shawinigan Ltd (London).
L-Sodium glutamate, from Ajinomoto (Japan), was obtained through the B.K. Trading Co. of Glasgow.

Other amino acids were bought from Cambrian Chemicals Ltd (London).
Agar was obtained from the Davis Gelatine Co. (N.Z.) Ltd.
Other chemicals were 'Analar' or the best grade available.

2. MEDIA

The composition of the media used is shown in Table 1.

2MBS is a 2x concentrate of a minimal basal salts solution (MBS) and formed the basis of many of the other media used. Minimal liquid (ML) and minimal agar (MA) were used as the routine minimal media. BLSG was the broth medium used for obtaining competent cultures for transformation and is a modification of that of Thorne and Stull (1966). Recipient cultures for transformation are diluted from BLSG into transformation medium (TM), a minimal medium required to induce competence. L-Broth was used for routine liquid culture in complete medium.

The amino acids for ML and MA were added as a 50x concentrate, and glucose, thiamine HCl, CaCl$_2$$\cdot$2H$_2$O, FeCl$_3$$\cdot$6H$_2$O and MnSO$_4$$\cdot$H$_2$O were always added separately from stock concentrated solutions.
Supplements for solid and liquid media were added as follows:

- adenine sulphate: 10 mg./l.
- thymine: 100 mg./l.
- amino acids: 20 mg./l.
- streptomycin sulphate: 1000 mg./l.
- cephalosporin C: 5 mg./l. was added to solid media for penicillinase induction on plates.

Sporulation medium (SS), was used for obtaining high yields of bacillary spores, and consisted of Difco potato extract - 10 g./l.; Casitone - 10 g./l.; yeast extract - 2 g./l.; Collins special salts (Collins and Kornberg, 1960) - 2 ml./l.
<table>
<thead>
<tr>
<th>Constituent</th>
<th>2MBS</th>
<th>ML</th>
<th>NA</th>
<th>BLSG</th>
<th>TM</th>
<th>L-Broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂HPO₄</td>
<td>29.3</td>
<td>14.65</td>
<td>14.65</td>
<td>14.65</td>
<td>14.65</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>11.0</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂PO₄</td>
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<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
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</tr>
<tr>
<td>Sodium citrate₂H₂O</td>
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<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
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<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
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<tr>
<td>FeCl₃·6H₂O</td>
<td>0.04</td>
<td>0.04</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
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<td>0.0005</td>
<td>0.001</td>
<td>0.0063</td>
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<td></td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
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<td></td>
<td></td>
<td></td>
<td>0.075 0.15</td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11.7 10</td>
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<td>Glucose</td>
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<tr>
<td>Thiamine HCL</td>
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</tr>
<tr>
<td>Glycerol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12.0</td>
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<tr>
<td>Difco nutrient broth</td>
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<td>8.0</td>
</tr>
<tr>
<td>L-Sodium glutamate</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Glycine</td>
<td>0.15</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>PVA (0-60 G)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Difco Tryptone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

Amounts of constituents are given in grams per litre.

All media were at pH7.
Bacillus licheniformis strains used for recipients in transformation were derived from the 9945A strains used in the transformation system of Thorne and Stull (1966). Transformable strains (9945A *pen*) give tough, red-brown, crinkled colonies, resistant to spreading by toothpicks (*pen* character). They were obtained by Thorne and Stull as colonial variants of the wild-type strain (ATCC 9945A.), which synthesizes large quantities of poly-D-glutamate (*pen*), giving mucoid colonies, the cells of which cannot be transformed.

The majority of the penicillinase mutants used were derived from *B. licheniformis* strain 749 (Kushner, 1960) and have been partially characterized, enzymologically and serologically (Dubnau and Pollock, 1965; Fleming and Hill, unpublished). Strain 749 produces a penicillinase indistinguishable from that synthesized by 9945A strains, judged by enzymological and serological tests (Fleming and Hill, unpublished). Strain 749 is poorly transformable (Dubnau and Pollock, 1965) and produces round, soft colonies. DNA made from 749 strains transforms 9945A *pen* recipients at the same frequency as DNA made from 9945A strains for all markers tested. Transformed cells may receive the *pen* character from either 9945A *pen* or 749 strains, suggesting that 749 and its derivatives carry the *pen* character, though expressed in a morphologically different form to 9945A *pen*.

**Nomenclature of strains.**

a) 9945A and its derivatives.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 9945A</td>
<td>Wild-type prototroph (<em>pen</em>, <em>pen</em> 1^+^<em>p</em>), ex Thorne.</td>
<td></td>
</tr>
<tr>
<td>9945A <em>pen</em></td>
<td>Transformable, rough colonial variant of 9945A.</td>
<td></td>
</tr>
</tbody>
</table>

The following mutants were derived in the *pen* state and were all transformable.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>M17</td>
<td>mde-1, ex Thorne.</td>
<td></td>
</tr>
</tbody>
</table>
M18  

M19  

7d  

3c  

7f  

Sa  

3b  

gb  

2N  

23A  

6T  

9T  

5L  

\( \text{non} \ i_{A}^{+} \).  

All these mutants, except 5L, were initially penicillinase inducible (\( \text{non} \ i_{A}^{+} \)).

In general the proposals of Demerec et al. (1966) for nomenclature have been followed. The gene symbols of Taylor and Trotter (1967) have been used except for the exceptions below. Where the biochemical nature of the mutation is now known an isolation number is given, and for the known enzymic blocks the designations below were given:

- \( \text{aur}^{+} \) is blocked at the ornithine transcarbamylase step in arginine biosynthesis,
- \( \text{trp}^{+} \) is blocked at anthranilate synthetase, and
- \( \text{illy}^{+} \) at the dehydrase step in isoleucine-valine biosynthesis.

\text{Ile}^{+} \) has been retained for a block at the threonine deaminase step.
(ilvA, in the nomenclature of Taylor and Trotter), to avoid confusion with the ilvD1 mutation. Ade-1 has been used rather than pur-1, because of common usage in this laboratory.

Penicillinase mutants (men) were originally given an isolation number (see Table 2), e.g. 749/110cen32. From the enzymological data and genetic analysis they were assigned a genotype in terms of P (structural gene), i (linked gene controlling inducibility) and r (additional regulatory genes).

Penicillinase mutations were introduced into 9945A men strains by transformation. The penicillinase genotype of 9945A strains is either given in full, e.g. 9T men i05P22 (often abbreviated to 9T/i05P22), or for the commonly used genotypes, a numbering system was used, e.g. for different 8a men genotypes:

- \(8a/1\) ade-1 ilvD1 men i05P
- \(8a/2\) men i05P72
- \(8a/3\) i05P
- \(8a/4\) i05P
- \(8a/5\) iA
- \(8a/6\) i05P22
- \(8a/7\) i05P72

b) 749 and its derivatives

A classification of the penicillinase mutants, derived from 749 is given in Table 2.
Table 2. Penicillinase mutants derived from *B. licheniformis* strain 749, used in this study (modified from Dubnau and Pollock, 1965). All the mutants except 749/110penC3 and 749penT9 were made by NTG treatment of the parent indicated. 749/110penC3 and 749penT9 were made using EMS as mutagen.

* Induction ratio = differential rate of penicillinase synthesis under standard induction conditions/differential rate of penicillinase synthesis without induction.

** CRM = specific precipitation with specific antiserum to wild-type penicillinase, by Ouchterlony plate test on surface growth on solid medium in the presence of inducer for inducible strains (Pollock, 1964).

*** Based on the phenotype classification of Collins et al. (1965).

* Penicillinase levels expressed as U./mg. dry wt. bacteria.

n.t. = not tested.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Penicillinase Activity*</th>
<th>Induction ratio*</th>
<th>CRM**</th>
<th>Phenotypic description***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninduced</td>
<td>Induced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>749</td>
<td>12-24</td>
<td>840-1400</td>
<td>35-117</td>
<td>++ Magno-inducible (pen i_p^+), wild-type</td>
</tr>
<tr>
<td>749/110</td>
<td>12-24</td>
<td>840-1400</td>
<td>35-117</td>
<td>++ his-1 atr-1</td>
</tr>
<tr>
<td>749/penC</td>
<td>3000-5000</td>
<td>3000-5000</td>
<td>1.0</td>
<td>++ Magno-constitutive</td>
</tr>
<tr>
<td>749/110penC3</td>
<td>3660</td>
<td>4120</td>
<td>1.02</td>
<td>++ Magno-constitutive</td>
</tr>
<tr>
<td>749penT9</td>
<td>19</td>
<td>1250</td>
<td>66</td>
<td>++ Magno-inducible at 30°C.</td>
</tr>
<tr>
<td>749/110penC3/22</td>
<td>0.05</td>
<td>0.05</td>
<td>-</td>
<td>++ Negative</td>
</tr>
<tr>
<td>749/penC/72</td>
<td>0.05</td>
<td>0.05</td>
<td>-</td>
<td>++ Negative</td>
</tr>
<tr>
<td>749/110penC3/16</td>
<td>3.8</td>
<td>2.6</td>
<td>0.7</td>
<td>++ Micro-constitutive</td>
</tr>
</tbody>
</table>

Penicillinase genotype

<table>
<thead>
<tr>
<th>Penicillinase genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>i_p^+</td>
</tr>
<tr>
<td>i_p^+</td>
</tr>
<tr>
<td>i_0^+</td>
</tr>
<tr>
<td>i_0^+</td>
</tr>
<tr>
<td>i_0^+_22</td>
</tr>
<tr>
<td>i_0^+72</td>
</tr>
<tr>
<td>i_0^+_16</td>
</tr>
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<tr>
<td>749/110cen32</td>
</tr>
<tr>
<td>749/110cen31</td>
</tr>
<tr>
<td>749/110cen27</td>
</tr>
<tr>
<td>749/110cen14</td>
</tr>
</tbody>
</table>
4. TRANSFORMATION

a) Transformation Procedure

The final procedure adopted for obtaining competent cultures was a modification of that of Thorne and Stull (1966). Recipient cultures were grown from a spore inoculum (ca. $10^6$ spores) in BLSG (25ml.) in Erlenmeyer flasks (250ml.). Growth was at 37°C, on an orbital shaker (Callenkamp Ltd) at 250 rev./min. Under these conditions the doubling time was 50 min. and the cultures reached stationary phase after 12–14 hours. After 17–18 hours in BLSG (ca. $10^9$ cells/ml.), cells were diluted 30 times into TH (1–2ml.) in test-tubes (1.8 x 15 cm.). DNA was added immediately after dilution and the transformation mixture was incubated at 37°C for 60 min. on a reciprocal shaker (5cm. stroke, 100 strokes/min.). Transformation was stopped by the addition of DNAase (40 μg. for 15 min.), after which the culture was plated onto solid medium and incubated for 40–48 hours at 37°C, unless otherwise stated.

In the preliminary experiments (Section A of Results), the experimental conditions were varied and are given for each experiment. For all experiments described in Section B of the Results, the conditions above were used.

For all transformations a control culture containing DNAase pre-treated DNA (40 μg. for 15 min.) was used to test for revertants or contaminants. None of the markers used reverted at a significant frequency and revertants were not normally observed on transformant plates.

b) Selection and scoring of transformants

(1) Auxotrophic transformants

Auxotrophic transformants were selected by plating transformed cultures onto appropriately supplemented agar plates. Double transformants were scored either by replica plating or by direct selection.
for the double requirement.

(ii) **Scoring of penicillinase transformants**

Penicillinase transformants were scored by the penicillin/iodine plate test (Materials and Methods: 7b) and given a tentative phenotype. Representative samples of different classes of penicillinase transformant were purified and either assayed in liquid culture or retested on solid media to test the original assignment of level. Penicillinase transformants producing more than 15 U./mg. could be picked directly off penicillin/iodine stained plates and re-streaked out. Colonies producing lower levels of penicillinase often did not survive the staining procedure, and were therefore picked from replica plates made before staining.

Up to 700 colonies per plate can be scored with this test under optimal conditions, i.e. the majority of colonies producing low levels of penicillinase, with the minority producing higher levels. When a large number of high level penicillinase producers were expected, the numbers of colonies per plate were reduced proportionately.

The original assignment of penicillinase level by the plate test was found to be correct in greater than 90% of cases, when the strain was re-tested in liquid culture.

(iii) **Streptomycin resistant (str\(^R\)) transformants**

Transformed cultures were plated onto a suitable medium lacking streptomycin. The plates were incubated for 4 hours at 37\(^\circ\)C. to allow expression of the new genotype and an agar overlay (3ml.) containing streptomycin to a final concentration of 1mg./ml. was then added. Streptomycin resistant transformants were counted after 40-48 hours at 37\(^\circ\)C.

(iv) **Pen\(^+\) transformants**

Pen\(^+\) transformants, could be directly counted on transformant
plates because of their distinctive colonial morphology.

c) DNA preparation

(i) Partially purified DNA

DNA for transformation was prepared either by the method of Marmur (1961) or by a phenol extraction procedure (Saito and Miura, 1963).

Cells for DNA were grown in L-broth supplemented with glycerol (0.25%) which inhibits sporulation (Thorne and Stull, 1966), and were harvested in late exponential phase (5-10 x 10^8 cells/ml.). The pellet was washed and resuspended in saline-EDTA (0.15N NaCl + 0.1M EDTA, pH 8.0). Lysis was by freeze-thawing (3-5 cycles), followed by lysosyme (0.2 mg/ml. for 50 min.) and sodium dodecyl sulphate (2% for 15 min. at 60°C). The lysates obtained by this procedure were then deproteinised and the DNA extracted according to the method of Marmur or Saito and Miura. After two deproteinization steps DNA (+RNA) were precipitated with ethanol and resuspended in sterile NaCl (2M). No attempt was made to remove RNA that precipitated with the ethanol. DNA was stored in NaCl (2M), saturated with chloroform, at 4°C. Under these conditions DNA could be stored for at least a year without significant loss of biological activity.

(ii) Crude lysates

Crude lysates of a number of strains were also used as DNA sources in transformation and were shown to give comparable recombination data to DNA prepared by other methods. Lysates were prepared by rapid freeze-thawing (3-5 cycles) of late exponential phase cultures grown in L-broth (1-2ml.) to which saline-citrate (0.15N NaCl, 0.015M trisodium citrate; pH 7.0) had been added after growth.

Strains for such lysates were restricted to 749-type or 9345A revertants which are non-transformable under the conditions used.
Strains for lysates also had auxotrophic requirements, so that unlysed cells could neither grow on transformant plates nor be transformed.

Usually 0.01 ml. of lysate was equivalent to about 0.1 µg. of purified transforming DNA. Lysates were stored frozen at -15°C, and were kept for a month, after which biological activity was significantly decreasing.

**d) Estimation of DNA**

DNA was estimated chemically by the method of Burton (1955). The amount of DNA estimated in this way was not found to be a reliable measure of biological activity. Therefore amounts of DNA for use in transformation were chosen by biological activity rather than chemical concentration.

### 5. ROUTINE CULTURAL CONDITIONS

Unless otherwise stated all cultures were grown at 37°C. Liquid cultures were grown in Erlenmeyer flasks filled to 1/10 of their total volume. Aeration was by shaking in an orbital incubator at 250 rev./min. Test-tube cultures were grown in tubes (1.6 x 15 cm.) on a reciprocal shaker (3 cm. stroke, 100 strokes/min.). Rough (men) strains were always grown in the orbital shaker, where the high aeration minimized clumping of the cells.

Growth in liquid culture was determined by measuring the absorbance of the culture at 675 µm. (2mm. path length) in a Unicam SP600 spectrophotometer. Absorbances were converted to mg. dry wt. bacteria/ml. by reference to a standard curve.

The relationship, 1 mg. dry wt. bacteria is equivalent to 10^9 bacterial cells, has been assumed throughout.

Growth on solid media was in plastic Petri-dishes (9 cm. diameter) containing about 20 ml. agar. Cultures (0.02-0.1 ml.) were spread on
agar plates with sterile Pasteur-pipettes bent into suitable shape for spreading immediately before use. This procedure was to avoid contamination from bacillary spores that often arises when a glass spreader, repeatedly sterilised in alcohol, is used for spreading plates.

Serial dilutions were made in ML and viable counts were made on ML plates supplemented with necessary nutritional requirements.

Stock cultures were usually maintained as spores, suspended in sterile distilled water. Strains for spores were grown in SS medium for a week or longer after which most viable cells had been converted into spores and much of the cell debris had autolysed. Spores were harvested and washed about 12 times with sterile distilled water, heat-activated at 65°C, for 60 min. and stored at 4°C, at a concentration of $10^9-10^{11}$ spores/ml. Some strains were maintained as spores in SS agar (0.75%) in Bijou bottles. Stocks kept in this way were more likely to revert, and had to be sub-cultured periodically. Spores maintained in water were stable indefinitely.

6. MUTAGENESIS

a) Mutagenic treatments

(i) UV irradiation of spores

About $10^7$ B. licheniformis spores in distilled water (1ml.) were irradiated in a glass Petri-dish for 30 sec., at a distance of 30cm. with a 150 watt UV lamp (Hanovia, Slough). Survival after this treatment was about 1%. Irradiated spores were either diluted into L-broth and grown up before plating, or plated directly onto a rich solid medium.

(ii) N-Methyl-N'-nitro-N-nitrosoguanidine (MNG) as mutagen

The method of Adelberg et al. (1965) was used for MNG treatment of vegetative cells to obtain auxotrophs.
Mutations to higher penicilllase level were obtained in the following way. A late exponentially growing culture (in L-broth) was diluted 20 times into ML supplemented with L-broth (0.04%) and grown for 45 min.; NGT was then added to a final concentration of 20 μg./ml. Incubation was continued for a further 60 min., after which the culture was harvested and resuspended in L-broth. The culture was plated out after 5 hours further growth at 37°C.

This treatment gave 1-2% auxotrophs and 0.05-1% magno-constituives from a magno-inducible strain. Auxotrophs were detected by plating onto MA supplemented with nutrient broth (100 μg./ml.), followed by replication onto MA. Penicillinase levels were scored on MA plates, with about 500 colonies per plate. Numbers of penicillinase revertants were compared to the numbers of auxotrophs obtained for each strain mutagenized, enabling a semi-quantitative estimate of the penicillinase reversion (i.e. mutation to higher penicillinase level) frequency.

This procedure was used for investigating the types of penicillinase level generated by mutagenesis of putative penicillinase regulatory gene mutations.

b) Selection and scoring of mutants

(1) Auxotrophic mutants

A thymine requiring mutant (thy-) was made according to the method of Okada et al. (1962). NGT mutagenization was followed by growth in minimal medium supplemented with aminopterin (100 μg./ml.) and thymine (100 μg./ml.). From 10 cultures treated in this way, 4 thy- mutants were obtained, all of which grew on greater than 15 μg./ml. of thymine. One of these (thy-1) was used for genetic analysis.

Other mutants for specific requirements were selected by a D-cycloserine enrichment technique, using the method of Curtiss et al. (1965). Penicillin selection is difficult with B.licheniformis.
because of the extracellular penicillinase produced. D-cycloserine, like penicillin, only affects growing cells, and therefore can be used for mutant selection in an analogous way to penicillin.

(ii) Penicillinase mutants

Penicillinase mutants were scored by the iodine/penicillin plate test \textit{(Materials and Methods: 7b)}. High level penicillinase producers survive this treatment and can be picked from the plates following staining. Low-level penicillinase producers (<15 U./mg.) often do not survive this treatment; plates containing low level penicillinase producing colonies were replicated before staining, so that such colonies could be picked from the replicas.

The optimal number of colonies per plate for detecting penicillinase mutants was 300-700, depending on the penicillinase level of the majority of colonies; the higher the background penicillinase level, the fewer colonies per plate, could be scored accurately.

7. ESTIMATION OF PENICILLINASE ACTIVITY

a) Unit of penicillinase activity

One unit (U.) of penicillinase hydrolyses 1 \textmu{}mole of penicillin per hour at 30\textdegree{}C. and pH 7.0, in the presence of excess substrate, (Pollock, 1950).

b) The spectrophotometric Perret assay (J.F. Collins, unpublished) for quantitative estimations in liquid culture

(i) Introduction

The Perret assay (Perret, 1954) uses excess iodine strongly buffered at pH 4.2 to stop the enzymic reaction. Penicilloic acid, the product of penicillin hydrolysis by penicillinase, reduces iodine, rendering it colourless; unreacted iodine is determined by titration against thiosulphate. The difference between the assay and a blank
containing inactivated enzyme (pH4.2/I₂) gives a direct measure of enzymic activity.

The spectrophotometric modification uses identical assay conditions, but rather than estimating the excess iodine titrimetrically, the absorption difference between sample and blank is measured spectrophotometrically. This method is very rapid, and removes the accumulative errors introduced by taking the difference of two titration measurements.

(ii) Reagents
Stock iodine: 0.32 N, iodine

1.2 N, KI

Acetate buffer (2M.), pH 4.2

Penicillin solution: benzylpenicillin (2.4 mg./ml.), in phosphate buffer (0.1 M.)
and pH 7.0

Stock iodine was diluted 1/20 in acetate buffer for use in the assay.

(iii) Assay procedure
Assays were done at 30°C. and the incubation mixture consisted of:

2.5 ml. penicillin solution

0.5 ml. penicillinase (e.g. bacterial culture)

Reaction was allowed to continue for a known time (10-50 min.) after which reaction was stopped by the addition of the iodine/acetate solution (5 ml.). A control containing penicillin but no enzyme was incubated under identical conditions and after the addition of iodine/acetate, an amount of enzyme equal to that in the unknown was added. The unknown and control were incubated for a further 10 min. to allow complete reaction of penicilloic acid with the iodine. The absorption difference between unknown and control was measured at 499 μm. in a
double-beam spectrophotometer (Beckman DB.).

(iv) Calculation of penicillinase activity

Activity (U./ml.) =

\[ \Delta \text{absorbance at } 499 \text{ nm (1 cm. incubation x convert time x enzyme volume to path length x total volume x factor to convert factor to convert to 1 hour 1 ml).} \]

The wavelength at which the absorbance difference was measured \(499 \text{ nm}\) was empirically chosen so that the above relationship would be true (J.F. Collins, personal communication).

Penicillinase activities (U./ml.) were converted to specific activities (U./mg. dry wt. bacteria) by measuring the absorbance of the culture at 675 nm (2 mm. cell) and converting the absorbance to dry weight of bacteria by reference to a standard bacterial dry wt./absorbance curve.

Cultures for penicillinase assay were grown with shaking L-broth at \(37^\circ\text{C.}\), unless otherwise stated. Cultures waiting assay were kept frozen or in an ice-bath.

Cephalosporin C (2 \(\mu\)g./ml.) was used to induce cultures when desired; inducer was added to cultures at about 0.1 mg. dry wt. bacteria/ml. and growth allowed to continue for 2.5 hours, when the culture had reached 0.5-1 mg./ml. The growth of the culture (measured by absorbance at 675 nm) and its penicillinase content were measured at this time and specific activity of penicillinase (U./mg. dry wt. bacteria) determined. Cephalosporin C did not significantly affect growth of any of the strains tested. Uninduced cultures were assayed after growth had reached 0.5-1 mg. dry wt. bacteria/ml.

The induction ratio (I.R.) is defined as:

\[ \frac{\text{specific activity of penicillinase after induction}}{\text{specific activity of penicillinase without induction}} \]
under the induction conditions used.

(v) Accuracy of penicillinase determinations

The above assay gave reproducible results for a given enzyme sample, i.e. ±5% in the range 1-100 U./ml. and ±10% at higher levels. The assay was not used for penicillinase activities less than 1 U./ml.

Day to day variations in level for a given strain were much greater: ±25% for uninduced levels and ±30% for induced levels. The major variations in specific activity were probably due to the inaccuracies in correlating culture turbidities with bacterial dry wt. due to slight clumping, production of pigments, etc. Variations in induced level might well be due to the different physiological state of the culture at the time of induction.

Where possible assays were confined to 749 or 9945A pep^+ strains, as non strains tend to clump, especially in the later stages of growth, making accurate correlation of culture growth to bacterial dry wt. impossible.

c) Semi-quantitative estimation of penicillinase produced by bacterial colonies on solid medium. The iodine/penicillin plate test.

Penicillinase produced by bacterial colonies decolorizes iodine in the presence of penicillin. If starch or polyvinyl alcohol (PVA) are incorporated into the medium, an intense blue complex is produced in the presence of iodine. This complex is also decolorized by penicilloic acid. E. licheniformis produces an extracellular amylase and therefore PVA which cannot be hydrolysed (J.F. Collins, unpublished) was used in solid media, rather than starch.

A dilute iodine solution (1/4 stock iodine in water), containing sodium tetraborate (about 1 mg./ml.) to intensify the blue colour, was used to flood plates. After about 1 min. the iodine is replaced by a strong penicillin solution (about 10 mg./ml.) in phosphate buffer
(0.1 M, pH 7.0). After about a minute the plate is drained dry. Penicillinase producing colonies hydrolyze the penicillin and a sharp zone of decolorization appears around the colonies, against the intense blue background. The rate of decolorization is proportional to the amount of enzyme produced by the colonies.

The exact times of exposure of the plates to iodine and penicillin were varied to enable maximal sensitivity for the penicillinase levels expected.

Colonies producing as little penicillinase as 0.1 U./mg. dry wt. bacteria can be detected by this test. 2-fold differences in activity can be detected in the range of 1-30 U./mg. of penicillinase and 5-10 fold differences at other levels.

8. SEROLOGICAL TESTS: THE OUCHTERLONY PLATE TEST

The production of immunologically cross-reacting material (CRM) was tested by the Ouchterlony plate test on bacterial surface growth (Pollock, 1954).

NA plates were inoculated with streaks (8) of standard and test strains, arranged radially around a central well containing anti-wild-type penicillinase antisera (prepared by J. Fleming). Extracellular penicillinase diffusing from the bacterial growth precipitates with the antisera diffusing from the central well and distinct precipitin lines are seen for CRM+ strains. Induction where necessary was with 7-ACA (7-aminocephalosporanic acid) placed in the central well. To get a precipitin line, the protein equivalent to 200-400 U. of wild-type penicillinase needs to be produced per mg. dry wt. bacteria.

9. CONSTRUCTION OF STRAINS BY TRANSFORMATION

Multiple-marked strains can be constructed relatively easily by
transformation, using highly saturating DNA concentrations. Under these conditions greater than 10% co-transfer of unlinked markers can be obtained.

Auxotrophic requirements were introduced into strains by transformation at high DNA concentrations and were scored by replica plating after growth on a suitably supplemented solid medium.

Penicillinase mutations were introduced into strains by selecting for a suitable marker, followed by scoring for the double transformant. Penicillinase mutations could also be introduced without selection when recipient cells were exposed to very high DNA concentrations for a long time (2-5 hours). Under optimal conditions, 10% of a recipient culture could be transformed for a penicillinase marker without selection (see results).

10. PROTOCOL OF PENICILLINASE MAPPING EXPERIMENTS AND DETERMINATION OF RECOMBINATION VALUES

For mapping experiments penicillinase transformants were obtained by selecting for the loosely linked markers ilvD1 or ilac-1 or for the unlinked marker ilv-1. Transformants repaired for one of these markers were now scored for penicillinase transformants by the iodine/penicillin plate test. Using non-saturating DNA concentrations, 2-10% of ilv+ or ilac+ transformants were also transformed for penicillinase.

It was shown experimentally, that under these conditions the majority of penicillinase recombinants arising were the result of the interaction of a single transforming DNA molecule with the penicillinase region of the recipient chromosome.

The iodine/penicillin plate test allows the direct scoring of a wide range of penicillinase phenotypes (10,000 fold range) on the transformant plates. The most convenient procedure is to use recipients
with as low level of penicillinase as possible so that the majority of transformed colonies will not decolorise the iodine/PVA complex. In this way it is very easy to identify large numbers of donor and recombiant-type colonies, producing higher penicillinase levels. In crosses in which donor and recombiant phenotypes were distinguishable, it was possible to determine the recombination frequencies directly by comparison of the numbers of donor and recombiant phenotype. In other cases where the recombination frequency could not be deduced directly it was determined by reference to a cross done under the same conditions, but using a donor with distinguishable phenotype (Nester and Lederberg, 1961). The linkage between neu and ilvD1 or ile-1 is so low that it can be disregarded when investigating recombination within the penicillinase region (Results: B1).

2- and 3- factor crosses were used for penicillinase mapping experiments.

11. PROTEIN CHEMISTRY TECHNIQUES

The techniques used for investigating the protein chemistry of an altered penicillinase (Results: B4) were those currently used in this laboratory for structural studies of proteins (Asbeler and Brown, 1967; Asbeler and Meadway, 1969; Meadway, 1969). The details of such techniques are described when relevant in the appropriate results section.
RESULTS
A. PRELIMINARY STUDIES

1. INTRODUCTION

The aims of the initial studies were to get reproducible transformation frequencies using the transformation system of Thorne and Stull (1966), to transfer the penicillinase genes at high frequency and to develop a means of obtaining large numbers of penicillinase transformants, preferably by selecting transformants for a marker linked to the penicillinase genes.

2. PRELIMINARY TRANSFORMATION EXPERIMENTS

a) Repair of auxotrophic markers and the cotransfer of the genes for penicillinase and poly-D-glutamate synthesis (pen).

A number of transformable auxotrophic strains received from Thorne and Stull (1966) and mutants derived from these were tested for their ability to be transformed by DNA extracted from B.licheniformis strains 9945A and 749. All the auxotrophic markers initially tested could be transformed at high frequency by DNA prepared from either of the B.licheniformis strains, using either the Marmur method or a phenol extraction procedure for the preparation of the DNA.

A representative example of the results obtained for one particular recipient is shown in Table 3. The recipient, 3g/1 (ada-1

aaw71 pen iA p+) was grown in BLSG for 18 hours (1.1 x 10⁹ cells/ml.), diluted into TH (1/20) and incubated with shaking for 3.5 hours at 37°C., when DNA was added to the final concentration indicated. After incubation with shaking for a further 3 hours at 37°C., DNA'se was added (40 μg. for 15 min.) to terminate transformation. Transformants were selected on appropriately supplemented plates and numbers of penA + and pen iA p+ double transformants were scored after the transformants...
Table 3. Preliminary transformation experiments using an ade-1 <i>argF1</i> pen 1<sup>+</sup> recipient (3g/1). The results are compiled from two transformation experiments done under identical conditions. The first experiment used DNA prepared by the Marmur method and the second used DNA prepared by the phenol extraction procedure.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Donor DNA Concentration (μg./ml)</th>
<th>Transformants/ml.</th>
<th>Cotransfer frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extraction procedure</td>
<td>ade&lt;sup&gt;+&lt;/sup&gt;</td>
<td>arg&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>9945Apen&lt;sup&gt;+&lt;/sup&gt; pen 1&lt;sub&gt;P&lt;/sub&gt;</td>
<td>Marmur</td>
<td>1.4</td>
<td>23000</td>
</tr>
<tr>
<td>9945Apen&lt;sup&gt;+&lt;/sup&gt; pen 1&lt;sub&gt;P&lt;/sub&gt;</td>
<td>Phenol</td>
<td>2</td>
<td>13100</td>
</tr>
<tr>
<td></td>
<td>DNAase pre-treated</td>
<td></td>
<td>&lt;10</td>
</tr>
<tr>
<td>749penC</td>
<td></td>
<td>1.0</td>
<td>19000</td>
</tr>
<tr>
<td>9945Apen&lt;sup&gt;+&lt;/sup&gt; pen 1&lt;sub&gt;P&lt;/sub&gt;</td>
<td>Phenol</td>
<td>2 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>7080</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>954</td>
</tr>
</tbody>
</table>

Viable counts of transformed cultures were 3-4 x 10<sup>7</sup> cells/ml.

n.s. = not scored.

Cotransfer frequency for ade<sup>+</sup> arg<sup>+</sup> = ade<sup>+</sup> arg<sup>+</sup> transformants/ml ade<sup>+</sup> transformants/ml.

*ade<sup>+</sup> arg<sup>+</sup> double transformants selected on unsupplemented solid medium.
had grown (40-48 hours at 37°C.).

Pre-treatment of DNA with DNA'se (40 μg. for 15 min.) led to complete loss of transforming activity. The ade-1 and argF1 markers could be transformed at comparable frequencies, as could most markers tested. DNA prepared by both methods and from both strains gave comparable biological activity, judged by numbers of transformants. Numbers of transformants fell with decreasing DNA concentration. Double transformants were obtained at the highest DNA concentrations, though the frequency of cotransfer fell with decreasing DNA concentration, suggesting ade-1, argF1 and pen to be unlinked.

Auxotrophic markers transformed in these preliminary experiments were: ade-1, argF1, arg-2, trp-2, tyr-1, his-1 (ex 749/110) and mut-2 (ex 749/81, Dubnau and Pollock, 1965).

It was repeatedly observed that the same recipient grown under apparently identical conditions could give transformation frequencies varying over a 50-fold range. Therefore the effective DNA concentration per competent cell will depend not only on the amount of DNA added to a recipient culture, but also on the proportion of competent cells in the culture.

b) Storage of competent cells

In an attempt to standardize the transformation conditions, recipient cells approaching competence in BLSG (16 hours) were frozen in liquid N2 in the presence of 15% glycerol (Jensen and Haas, 1963). On thawing, the cells were diluted immediately into TN, or grown for 2 hours in BLSG before dilution, and transformation attempted. In all cases transformation frequencies were very low, though the viable count had not dropped significantly. Therefore this technique was abandoned and fresh recipient cultures were prepared for each experiment.

c) Transformation of the penicillinase gene using a prototrophic
Because of the high transformation frequencies obtained (Table 3), an attempt was made to score *pen* transformants directly without selection, after transformation. Such a procedure would be useful for constructing strains isogenic except for different *pen* genotypes.

A 9945A *pen* *pen* i_{03} p_{22} recipient was transformed with a 9945A *pen* *pen* i^{+} p^{+} (9945A/WT) donor. The recipient was grown for 18 hours in BLSQ (1.5 x 10^9 cells/ml.), diluted into TH (1/20) and incubated for 3.5 hours when DNA was added to a final concentration of 14 µg./ml. The culture was incubated with DNA for 2 hours and transformation was terminated with DLI (40 µg. for 15 min.). Colonies were counted and scored for penicillinase level after 42 hours growth at 37°C on MA plates.

9.5% of the colonies were *pen*^{+} and 3.5% were *pen*^{+}. 14% of the *pen*^{+} colonies were *pen* i_{03} p^{+} and the rest *pen* i^{+} p^{+}. Therefore, using a saturating concentration of DNA and a prototrophic recipient, 14% recombination was obtained between the *pen* i_{03} and p_{22} loci. These results, showing a structural gene mutation and a regulatory locus conferring magno-constitutivity to be closely linked, confirm the results of Dubnau and Pollock (1965).

In a similar experiment in which the DNA concentration was reduced to 1 µg./ml., the proportion of *pen*^{+} transformants in the recipient population dropped to 2.3%, while the proportion of *pen* transformants had dropped to <0.4%.

Therefore such crosses, using very high DNA concentrations, are useful for constructing strains but are of little use for genetic analysis, where large numbers of penicillinase transformants need to be generated at non-saturating DNA concentrations.

The results also show that for this prototrophic recipient, at
least 10% of the cells are competent. The results with auxotrophic recipients show that in general a smaller proportion of the recipient population can be transformed, (e.g. see Table 3).

3. THE SEARCH FOR A MUTATION LINKED TO PENICILLINASE

a) Introduction

To undertake a detailed genetic analysis of the penicillinase genes, a linked marker is required, which when selected after transformation, will give a large number of pen double transformants. Linkage between markers can be either true linkage, where the markers are physically close and are often transformed and integrated on the same DNA molecule, or apparent linkage, which results from the integration of a number of DNA molecules at different sites on the recipient genome. Apparent linkage is usually observed at high DNA concentrations.

For genetic analysis, non-saturating DNA concentrations must be used to minimize the number of multiple interactions at the sites under study. It is therefore necessary to utilize true linkage for obtaining pen transformants for mapping purposes, though apparent (or pseudo-) linkage is a useful tool for constructing strains. A number of approaches used to obtain a linked marker are now described.

b) Generation of linked markers after nitrous acid treatment of transforming DNA

Nitrous acid treated transforming DNA has been used to generate linked markers in B.subtilis (Barat et al., 1965).

Wild-type B.licheniformis transforming DNA (9945A/WT) was treated with nitrous acid (Materials and Methods: 6a) and used to repair a penicillinase structural gene mutation in strain 9945A pen pen \( i_{c3} p_{22} \). The transformation conditions and DNA concentrations were the same as
those used for the same recipient in Section 2c. As well as the nitrous acid treated DNA, a control of unmethylated DNA was used to measure the competence of the recipient culture. Penicillinase transformants (i\textsuperscript{C\textsubscript{P}+} and i\textsuperscript{P+}) were scored after growth on complete medium and were tested for induced mutation by growth on minimal medium at 45\textdegree{}C.

The loss in biological activity after nitrous acid treatment was about 95%; 0.3\% of recipient cells were transformed to pen\textsuperscript{+} and a total of 8 pen transformants (all i\textsuperscript{P+}) were scored from about 12000 colonies examined. All 8 pen transformants grew at 45\textdegree{}C on minimal medium.

This technique would appear to be far more useful for generating linked markers, when transformants can be selected and so a large number can be tested for induced linked mutations (e.g. see Section 6a). The use of the penicillinase selection procedure of Dubnau and Pollock (1965), might have been profitably used to obtain more penicillinase transformants. However, as another procedure gave a marker linked to penicillinase (Section 5b), the selection procedure was not used.

c) A rational approach

Penicillin is thought to inhibit cell-wall biosynthesis in bacteria, probably at the transpeptidase step in gram-positive bacteria (Rogers, 1967). It has been proposed that penicillinase may have evolved from such an enzyme (e.g. see Citri and Pollock, 1966). If so, the penicillinase genes might be genetically linked to resistance markers to antibiotics which are known to inhibit cell-wall biosynthesis. D-cycloserine and O-carbamyl-D-serine are such antibiotics and are thought to inhibit the steps preceding the penicillin sensitive reaction in cell-wall biosynthesis (Curtiss et al., 1965; Lynch and Neuhaus, 1966; Rogers, 1967). D-cycloserine
B. licheniformis mutants, resistant to 50 μg./ml. of D-cycloserine, were isolated after UV irradiation of M17/I spores, followed by germination in liquid medium and growth on solid medium containing D-cycloserine. 80% of unmutagenized cells did not grow on 10 μg./ml. of cycloserine and 99% did not grow on 20 μg./ml. However D-cycloserine in this range of concentrations is only bacteriostatic (Curtiss et al., 1965) and moreover it was found to have an effective life in solid media of 1.5-3 days at 37°C. It was therefore impossible to select for D-cycloserine resistance after transformation, because sensitive cells could grow by the 42 hours required for transformants to appear. Therefore linkage to penicillinase could not be tested in this type of experiment. Resistance mutations to higher concentrations of D-cycloserine were not obtained. D-cycloserine resistant mutants were also resistant to O-carbaryl-D-serine (20 μg./ml.), but again the resistance character could not be selected because the lability of the antibiotic eventually allowed sensitive cells to grow.

Mutants resistant to higher concentrations of antibiotic need to be isolated, or a more stable antibiotic of similar action is required, if the linkage of penicillinase to these resistance markers is going to be investigated further.

d) Random testing of linkage between penicillinase and auxotrophic markers

Auxotrophic recipients were transformed with a prototrophic penicillinase constitutive DNA (9945A non\textsuperscript{+} non\textsuperscript{iA}P\textsuperscript{+}) at non-saturating concentrations (about 0.2 μg./ml.). The conditions used were those described in Section 2a, using J5/1 as a recipient. The linkage between penicillinase and most of the markers tested was less than 0.5%
Markers tested that showed no linkage to penicillinase were: ade-1, oxyF1, oxy-2, thr-2, his-1, tyr-1, met-1, met-2, leu-1 and nap.

However with an isoleucine-valine requirement (iiv), greater than 2% linkage to penicillinase was observed, when the linkage of ade-1 to nap was 10 times lower in the same experiment. This mutant was tested for its biochemical block by Dr C. Anagnostopoulos (Gif-sur-Yvette, France) and was shown to be blocked at the dehydrase step in isoleucine-valine biosynthesis. The mutant site was therefore designated iivD1 (Taylor and Trotter, 1967).

A. iivD1, A MUTATION LINKED TO PENICILLINASE

a) DNA dilution experiment to verify linkage

If the linkage observed between two markers results from the uptake and integration of the markers on the same transforming DNA molecule, the linkage should remain constant on DNA dilution, unless shearing of DNA occurs. If the linkage is the result of the uptake and integration of two DNA molecules, the linkage will fall on DNA dilution.

The effect of DNA dilution on the linkage of iivD1 and nap was investigated in the experiment described in Table 4.

The data are plotted graphically in Figure 1, the numbers of transformants at 0.8 µg./ml. DNA being normalised to 100 for all classes. It can be clearly seen that the linkage of nap to iivD1 is retained on dilution of the DNA, unlike the linkage of nap to ade which is lost on DNA dilution. It therefore seems likely that iivD1 and nap are transformed and integrated on the same DNA molecule.
**Table 4.**

Recipient: 8a/1; ade-1 ilvD1 pen i_A^+.

Donor: 9945A pen i_A^+.

<table>
<thead>
<tr>
<th>DNA concentration (µg./ml.)</th>
<th>ade^+</th>
<th>ade^+ i_A^+</th>
<th>ilv^+</th>
<th>ilv^+ i_A^+</th>
<th>*ade^+ ilv^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 x 10^-1</td>
<td>3.9 x 10^4</td>
<td>117</td>
<td>3.3 x 10^3</td>
<td>65</td>
<td>34</td>
</tr>
<tr>
<td>8 x 10^-2</td>
<td>1.5 x 10^4</td>
<td>15</td>
<td>690</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td><strong>8 x 10^-3</strong></td>
<td>1.9 x 10^3</td>
<td>0.4</td>
<td>73</td>
<td>2.8</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* Determined by replication of ade^+ transformants onto plates lacking isoleucine-valine.

** 5 ml. transformed culture plated for both ade^+ and ilv^+ transformants.

Experimental conditions were as described in Section 2a for 5g/1.
Figure 1. The effect of decreasing DNA concentration on numbers of ade-1, ilvD1, ade-1 ilvD1, ade-1 pen iA+p, and ilvD1 pen iA+p transformants, using 8a/1 as a recipient.
b) DNA competition experiment

If *ilyD1* ren double transformants result from the integration of a single transforming DNA molecule, the linkage should be retained in the presence of a competing DNA. An *ilyD1* ren *i0P*72 recipient was transformed with an *ilyD1* + ren *i0P* + DNA alone, and in the presence of a competing *ilyD1* ren *iP* + DNA. The results from the experiment are shown in Table 5.

Table 5.

Recipient: 6T/7; ade-1 *ilyD1* thy-1 ren *i0P*72

Donors: 749renC (*ilyD1* + ren *i0P* +)

*8a/1* (*ilyD1* ren *iP* +)

| Donor DNA | Transforms/0.6ml.
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
<td><strong>Concentration</strong></td>
</tr>
<tr>
<td>749renC</td>
<td>20 µl.</td>
</tr>
<tr>
<td>8a/1</td>
<td>40 µl.</td>
</tr>
<tr>
<td>749renC +</td>
<td>10 µl.</td>
</tr>
</tbody>
</table>

Numbers of thy+ transformants give a measure of biological activity for each of the DNA’s. The experimental conditions are those given in Materials and Methods: 4a for transformation procedure. In the culture transformed with both DNA’s (cross 3), all ren transformants were derived from the DNA able to repair *ilyD1* and it is therefore concluded that *ilyD1* and ren are transformed on the same DNA molecule.
5. FACTORS AFFECTING THE APPEARANCE OF TRANSFORMANTS

AND THE LINKAGE BETWEEN ilvD1 AND PENCILLINASE

a) Phased appearance of transformants and variable linkage between ilvD1 and pen.

To standardize transformation conditions, an attempt was made to determine the time of maximal competence after dilution of a recipient culture from BLSG into TM.

The recipient, 8a/1 (ade-1 ilvD1 pen i+p+), was grown in BLSG for 17 hours \((9 \times 10^8\) cells/ml.) and diluted 1/20 into TM (24 ml.), from which samples (2 ml.) were taken at 30 min. intervals and exposed to 9945A pen+ pen i+p DNA \((0.8 \mu g./mL.)\) for 15 min., followed by DNA'se \((40 \mu g.\) for 15 min.) and plating onto selective media. The results are shown in Table 6 and in Figure 2.

Numbers of ade+ transformants were a maximum immediately after dilution from TM, and they fell thereafter. Two peaks of ilv+ transformants were obtained, the smaller one after 30 min. in TM and the major one after 210 min. The linkage between ilvD1 and pen was variable, being highest when the numbers of ilv+ transformants were a maximum \((50\%\) linkage), and lowest when the numbers of ilv+ transformants were a minimum. A portion of the culture was exposed to DNA for the whole 330 min. period in TM; the numbers of transformants obtained, indicated that the DNA had lost transforming activity by later times in TM, probably, either because of its degradation by nucleases or because it has been completely utilized. Another possibility is that the effect of the continued presence of the DNA was to cause overall loss of competence. Numbers of ade+ ilv+ double transformants (selected by plating onto unsupplemented medium) were only found in significant numbers \((i.e., >1)\) in the first sample after dilution from BLSG, when 4 ade+ ilv+ transformants were selected \((0.25\) ml. culture plated). Three
Table 6. The effect on the appearance of transformants of the addition of DNA to a culture at different times after its dilution into TM.

Recipient: 8a/1; sde-1 ilvD1 pen i^p^+
Donor: 9945Apen^p^+ pen i_A^p^+

<table>
<thead>
<tr>
<th>Time of addition of DNA after dilution into TM (min.)</th>
<th>Transforms/ml.</th>
<th>Cotransfer frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ade^+</td>
<td>ilv^+</td>
</tr>
<tr>
<td>0</td>
<td>15000</td>
<td>95</td>
</tr>
<tr>
<td>30</td>
<td>6700</td>
<td>342</td>
</tr>
<tr>
<td>60</td>
<td>4800</td>
<td>163</td>
</tr>
<tr>
<td>90</td>
<td>3600</td>
<td>73</td>
</tr>
<tr>
<td>120</td>
<td>2400</td>
<td>49</td>
</tr>
<tr>
<td>150</td>
<td>1800</td>
<td>69</td>
</tr>
<tr>
<td>180</td>
<td>1100</td>
<td>108</td>
</tr>
<tr>
<td>210</td>
<td>760</td>
<td>685</td>
</tr>
<tr>
<td>240</td>
<td>432</td>
<td>155</td>
</tr>
<tr>
<td>270</td>
<td>381</td>
<td>58</td>
</tr>
<tr>
<td>330</td>
<td>109</td>
<td>96</td>
</tr>
<tr>
<td>0-330</td>
<td>27300</td>
<td>1020</td>
</tr>
</tbody>
</table>

DNA (0.8 µg./ml.) was added for 15 min. at the time indicated. 1 ml. of transformed culture was plated for each transformant class at each time, except for the 0-330 min. exposure to DNA (0.25 ml. plated).

<1/10^3 of the ade^+ transformants obtained from any 15 min. exposure to DNA, were also transformed for penicillinase. Ade^+ pen i_A^p^+ double transformants were not scored for the 0-330 min. exposure to DNA.
Figure 2. The effect on the appearance of transformants of the addition of DNA to an 8a/1 culture at different times after its dilution into TM.

- ade^+
- ilv^+
- ilv^+ pen i_Ap^+
of these transformants were also transformed for pen, i.e. ade\(^+\) ily\(^+\) pen \(i_{A}^{p+}\). Cotransfer of pen with ade-1 was low (<1/10\(^{3}\)) for any 15 min. exposure to DNA. Ade-1 ily\(^D1\) and ade-1 pen \(i_{A}^{p+}\) double transformants were not scored for the 330 min. exposure to DNA in TM.

This experiment was repeated a number of times and in all cases the ade\(^+\) peak was observed immediately after dilution into TM, while the early ily peak occurred either at 0 or 30 min. after dilution from BLSG. The second ily peak was seen between 180 and 270 min. after dilution into TM and always contained more ily transformants than the early peak. Linkage between ily and pen was always greatest when ily transformants were at a maximum. The linkage values for ily and pen were not as high in the subsequent experiments where the mean maximal value of linkage was 26% and for the 330 min. exposure to DNA was 5%.

The viable count during the 330 min. period remained constant (± 10%) until 210 min. after which it fell slowly till 330 min. (about 25% fall). Whether this constancy is due to an undividing, undying population or reflects a balance between division and death is not known.

The net change in DNA content of an 8a/1 culture throughout the 330 min. period in TM was measured. 8a/1 was grown in BLSG (25 ml.) and diluted into TM (500 ml.) under exactly the same conditions as for the transformation experiment. Samples were taken at 45 min. intervals and the cells were harvested and washed once with fresh TM. DNA was extracted with perchloric acid and estimated by the method of Burton. The net DNA increase by the end of the 330 min. period was 22%. It seems reasonable to conclude that the physiological state of the culture used for the DNA estimations was the same as that used for transformations, though the total volume of TM incubated for transformations was 1/20 of that used for the DNA estimations.
The differential appearance of transformants suggests that the 8a/1 culture has become phased in some way and it is evident that one cannot define competence in this system in the way that it is applied to other transformable bacteria. 'Competence' for difference markers appears at different times. Such behaviour can be explained if there are different competent populations which are capable of being transformed for different markers at different times. Another possibility is that competent cells can distinguish DNA's carrying different markers; the uptake of different markers occurring only at specific times. Both of these explanations seem rather unlikely. A third possible explanation is that the competent 8a/1 cells are fortuitously synchronized and the phased appearance of transformants reflects the integration of transforming DNA at a specific position on the replicating genome at any given time. This hypothesis is discussed with reference to the results presented here and to the results obtained from some other systems in the Discussion.

Whatever the nature of this behaviour, it was evident that a detailed investigation would be required to study it further and it was therefore decided to undertake experiments to optimize and standardize the transformation conditions, taking into account the phased appearance of transformants and variable linkage.

b) Other factors affecting the appearance of transformants

(i) **The effect of time of dilution from BLSG on the appearance of transformants**

The effect of time of dilution of a recipient culture from BLSG into TM was investigated. The recipient used was 8a/1 and the results are shown in Table 7.

Transformants can be obtained after dilution from BLSG between 13 and 19 hours, though no transformants were obtained after dilution at
Table 7. Effect of time of dilution from BLSG into TM on the appearance of transformants.

Recipient: 8a/1; ade-1 ilyD1 pen i_A^+ Donor: 9945A pen i_A^+.

<table>
<thead>
<tr>
<th>Time of dilution from BLSG to TM (hours after inoculation)</th>
<th>Absorbance at 675 μm. (2 mm. cell)</th>
<th>0 min.</th>
<th>30 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>0.018</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>0.15</td>
<td>758</td>
<td>188</td>
</tr>
<tr>
<td>14</td>
<td>0.23</td>
<td>550</td>
<td>180</td>
</tr>
<tr>
<td>15</td>
<td>0.28</td>
<td>920</td>
<td>163</td>
</tr>
<tr>
<td>16</td>
<td>0.34</td>
<td>1190</td>
<td>225</td>
</tr>
<tr>
<td>17</td>
<td>0.38</td>
<td>1875</td>
<td>235</td>
</tr>
<tr>
<td>18</td>
<td>0.40</td>
<td>1590</td>
<td>285</td>
</tr>
<tr>
<td>19</td>
<td>0.40</td>
<td>668</td>
<td>154</td>
</tr>
</tbody>
</table>

Transformants/ml.:
- ade^+ ily^+ i_A^+
- ade^+ ily^+ i_A^+
- ade^+ ily^+ i_A^+
- ade^+ ily^+ i_A^+
- ade^+ ily^+ i_A^+

Cultures were diluted 1/20 from BLSG at the time indicated. Cells in TM were exposed to DNA (0.8 μg./ml.) for 15 min. either immediately after dilution, or 30 min. later. Transformations were terminated by DNA'se (40 μg. for 15 min.). 1 ml. of culture was plated for each class of transformants.
9 hours. Few transformants (<50/ml. of either ade<sup>+</sup> or ilv<sup>+</sup>) were obtained if DNA was added directly to a BLSG culture at 17 hours without dilution into TM; the step-down into TM seems to be necessary to achieve good transformation frequencies. At all times except 14 hours, the numbers of ade<sup>+</sup> transformants were greater for the 0 min. TM sample. This effect was most marked after 17 or 18 hours growth in BLSG.

Numbers of ilv<sup>+</sup> transformants from the 0 min. and 30 min. TM samples did not vary significantly. It was not practicable to continue sampling after longer than 30 min. incubation in TM, because of the large numbers of experimental manipulations already involved.

Though similar experiments were not done using different recipient cultures, it was observed in other experiments that some recipients could only be transformed after at least 17 hours in BLSG (e.g. 7d; ade<sup>-1</sup> tyr<sup>-1</sup>).

In subsequent transformation experiments, cultures were diluted from BLSG between 17 and 18 hours (9-15 x 10<sup>3</sup> cells/ml.), as this seemed the optimal time for maximal numbers of transformants for the recipients tested.

(ii) Effects of growth supplements on the appearance of transformants.

The effects of the addition of different growth supplements to BLSG and TM on transformation are shown in Table 3. The effects due to the lack of some metal ions in BLSG are also shown.

The addition of either adenine or isoleucine-valine to BLSG had little effect on numbers of ade<sup>+</sup> and ilv<sup>+</sup> transformants. Growth was increased slightly if either adenine or isoleucine-valine was added to BLSG, the addition of both gave a roughly additive increase (35% increase over unsupplemented growth). From these results it is impossible to conclude what was limiting growth.
Table 8. Effect of different growth conditions on the appearance of ade-1 and ilvD1 transformants.

Recipient: 8a/1; ade-1 ilvD1 pen i^R. Donor: 9945A pen i^R.

<table>
<thead>
<tr>
<th>BLSG supplemented with:</th>
<th>TH supplemented with:</th>
<th>Transforms/ml. ade^+</th>
<th>Transforms/ml. ilv^+</th>
<th>Absorbance of BLSG at 675 nm. (2mm. cell) culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>5500</td>
<td>408</td>
<td>0.355</td>
</tr>
<tr>
<td>ade (10 µg./ml.)</td>
<td>-</td>
<td>5280</td>
<td>390</td>
<td>0.42</td>
</tr>
<tr>
<td>ilv (20 µg./ml.)</td>
<td>-</td>
<td>5380</td>
<td>386</td>
<td>0.41</td>
</tr>
<tr>
<td>ade + ilv</td>
<td>-</td>
<td>5340</td>
<td>430</td>
<td>0.48</td>
</tr>
<tr>
<td>-</td>
<td>ade (10 µg./ml.)</td>
<td>1200</td>
<td>61</td>
<td>0.355</td>
</tr>
<tr>
<td>-</td>
<td>ilv (20 µg./ml.)</td>
<td>4900</td>
<td>390</td>
<td>0.355</td>
</tr>
<tr>
<td>-</td>
<td>ade + ilv</td>
<td>1230</td>
<td>58</td>
<td>0.355</td>
</tr>
</tbody>
</table>

BLSG lacking:

- Fe/Mn - Transformation frequencies 0.12 (culture lumpy)
- Ca - reduced by at least 90% for 0.34
- Fe/Mn/Ca - all classes of transformant. 0.09 (culture lumpy)

BLSG culture was diluted (1/20) after 17 hours growth. The absorbance of the cultures at this time is shown. DNA (about 0.5 µg./ml.) was added immediately after dilution into TH for 60 min.
The addition of isoleucine-valine alone to TM had little effect on transformation. The presence of an appreciable concentration of adenine (10 μg./ml.) in TM, inhibited the appearance of transformants: 78% drop in ade<sup>+</sup> transformants, 86% drop in ilv<sup>+</sup> transformants.

The lack of added Fe/Mn to BLSG severely affected growth and cultures were lumpy. Transformation frequencies were greatly reduced: <40 ade<sup>+</sup> transformants/ml. The lack of Ca did not affect growth appreciably, though transformation frequencies were reduced by about 90%.

c) Conclusions

With 8a/1 as recipient, maximal numbers of ade-1 and ilvD1 transformants were obtained after 17-18 hours growth in BLSG (9-15 x 10<sup>8</sup> cells/ml.) before dilution into TM. The appearance of ade-1 and ilvD1 transformants, after incubation with DNA at different times in TM, is phased and the linkage between nen and ilvD1 is variable, being greatest when numbers of ilv<sup>+</sup> transformants are highest.

Neither lack of adenine nor isoleucine-valine seemed to be limiting growth in BLSG. Addition of either adenine or isoleucine-valine to BLSG did not appreciably affect transformation frequencies. However, the presence of adenine (10 μg./ml.) in TM did reduce the numbers of transformants. The metal ions, Fe/Mn and Ca are required for transformation, though Ca was not necessary for growth. It was also observed in experiments not reported here that a 1/30 dilution from BLSG into TM resulted in slightly higher transformation frequencies, than when BLSG cultures were only diluted 1/20. Another advantage of the higher dilution from BLSG is that there is less background growth from untransformed cells on transformant plates.

As a result of these experiments, a final transformation procedure was adopted (Materials and Methods: 4a). BLSG cultures were grown for
17-18 hours and diluted 1/30 into TM containing DNA. Transformation was usually terminated after one hour.

6. OTHER MARKERS LINKED TO ILVD1 AND PENICILLINASE

Markers linked to ilVD1 may be more closely linked than ilVD1 to penicillinase. Two procedures were used for obtaining linked markers to ilVD1.

a) Generation of a marker linked to ilVD1, after transformation with nitrous acid treated DNA

Nitrous acid treated transforming DNA was used to repair ilVD1 in 3a/1 (ade-1 ilVD1 pen 1+ p+). After transformation (5 μg./ml. DNA for 3 hours), ilV+ transformants were selected by growth at 37°C on solid medium supplemented with nucleotides, vitamins and all the L-amino acids except isoleucine and valine. Transformant ilV+ colonies were then replicated onto minimal medium at 45°C to score for mutants. From two experiments, one mutant, responding to tryptophan and anthranilate, was obtained. This mutant was designated trpE1 (Taylor and Trotter, 1967) and its linkage to ilVD1 and pen was investigated in the following transformation experiments.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>*Cross</th>
<th>Transformants/ml.</th>
<th>Linkage between ilVD1 and trpE1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2N/1</td>
<td>8b/6</td>
<td>ilVD1 x trpE1</td>
<td>trp+ ilV+ 790, trp+ ilV+ 792, trp- ilV+ 98</td>
<td>11%</td>
</tr>
<tr>
<td>2. 8a/1</td>
<td>2N/6</td>
<td>trpE1 x ilVD1</td>
<td>ilV+ trp+ 3840, ilV+ trp+ 3610, ilV+ trp- 230</td>
<td>6%</td>
</tr>
</tbody>
</table>

* Donor first.

Penicillinase was not linked to trpE1: 2/890 trp+ transformants
were also transformed for pen (pen⁻) and both were found to be ily⁻.  
5% of ily⁺ transformsants (cross 2) were also transformed for penicillinase. It can therefore be concluded that tyrD1 is weakly linked to ilyD1, and maps on the side of ilyD1 distal to penicillinase. In B. subtilis, ilyD and ile mutations map close to the genes coding for the tryptophan biosynthesizing enzymes (Barat et al., 1965; Kelly, 1967; Dubnau et al., 1968; Appendix III).

b) Other markers linked to ilyD1

(i) Introduction

Genes specifying a given biochemical pathway are often linked and so an investigation of other mutants blocked in the same pathway may lead to a linked mutation. In addition it is known that B.licheniformis is related to B.subtilis and has a similar genetic map (Tyeryar et al., 1968); therefore examination of the more complete B.subtilis genetic map should suggest markers that might be linked to ilyD1 (B.subtilis has no penicillinase). The ilyD locus in B.subtilis is located near to the terminus of the replication map and is linked to ile, met and thy loci (Dubnau et al., 1967; Appendix III).

(ii) The linkage of ile-1 to ilyD1 and pen

The linkage of ile-1 to ilyD1 and penicillinase was investigated in the following transformation experiment.

<table>
<thead>
<tr>
<th>Table 10.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recipient</strong>: 8a/6; ade-1 ilyD1 pen i₃₃P₂₂⁻</td>
</tr>
<tr>
<td><strong>Donor</strong>: 9b/1; ade-1+ ilyD1+ ile-1 pen iₚ⁺</td>
</tr>
<tr>
<td><strong>Transformants/ml.</strong></td>
</tr>
<tr>
<td>ily⁺ile⁺</td>
</tr>
<tr>
<td><strong>pen⁻</strong></td>
</tr>
<tr>
<td>i₃₃P⁺</td>
</tr>
<tr>
<td>iₚ⁺</td>
</tr>
<tr>
<td>* penicillinase negative i.e. iₚP₂₂⁻</td>
</tr>
</tbody>
</table>


DNA concentration was non-saturating (about 0.1 μg./ml.) and the transformation conditions are those described in Materials and Methods: 4a.

The proportion of \textit{ialy} \textit{ile} \textsuperscript{+} transformants among the \textit{ialy} \textit{ile} \textsuperscript{-} was determined by replication.

\textit{IlyD1} and \textit{ile} \textsuperscript{-} are 57\% linked, while the higher penicillinase linkage to the \textit{ialy} \textit{ile} \textsuperscript{-} class of transformants suggests that recombination between \textit{ialyD1} and \textit{ile} \textsuperscript{-} decreases the frequency of incorporating penicillinase on the same DNA fragment. This data is suggestive that the order is \textit{ialyD1 - ile} \textsuperscript{-} - pen, though the low linkage between \textit{ialy/ile} and penicillinase makes interpretation of such results difficult.

Unfortunately the reciprocal cross cannot be carried out because on selection for \textit{ile} \textsuperscript{+}, all \textit{ialy} transformants will be lost. More experiments showed the linkage of \textit{ialyD1} to \textit{ile} \textsuperscript{-} to vary between 50-80\% at non-saturating DNA concentrations, and the linkage of \textit{pen} to \textit{ialy/ile} was highest when the linkage of \textit{ialyD1} to \textit{ile} \textsuperscript{-} was highest.

In experiments in which an \textit{ile} \textsuperscript{-} recipient was repaired with a prototrophic \textit{pen} marked DNA the linkage between \textit{ile} \textsuperscript{-} and \textit{pen} was found to be 2-10\%, i.e. of the same order as that between \textit{ialyD1} and \textit{pen}. From these experiments it was not possible to deduce if \textit{ile} \textsuperscript{-} or \textit{ialyD1} was nearer to \textit{pen}.

In \textit{B. subtilis} an \textit{ile} \textsuperscript{-} mutation is 40\% linked to an \textit{ialyD} mutation by transformation (Barat \textit{et al.}, 1965).

(iii) The linkage of \textit{met} \textsuperscript{-1}, \textit{thy} \textsuperscript{-1} and the 'aromatic' genes to \textit{ialyD1}.

Because of the linkage of \textit{tryE1} to \textit{ialyD1}, other mutants impaired in aromatic biosynthesis were tested for linkage to \textit{ialyD1} and \textit{tryE1}.

The mutations tested and linkage values obtained are shown in Table 11.
I'bitation in
relation in donor
recipient

<table>
<thead>
<tr>
<th>Mutation in</th>
<th>Mutation in donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>recipient</td>
<td>trpE1</td>
</tr>
<tr>
<td>trpE1</td>
<td>-</td>
</tr>
<tr>
<td>tyr-1</td>
<td>19</td>
</tr>
<tr>
<td>trp-2</td>
<td>n.t.</td>
</tr>
<tr>
<td>ilyD1</td>
<td>6</td>
</tr>
</tbody>
</table>

n.t. = not tested.

Numbers are % linkage at non-saturating DNA concentrations.
The only marker to show linkage to nen was ilyD1.

These results show mutations impairing aromatic amino acid
biosynthesis to be linked, as in B. subtilis (Appendix III). The linkage
values suggest that trpE1 is closer than tyr-1 to ilyD1. In B. subtilis,
trpE and tyr are at opposite ends of the 'aromatic' cluster of genes,
tyr and his being nearer than trpE to ilyD. It therefore seems possible
that the aromatic cluster is orientated in opposite directions in the
two organisms. Three factor reciprocal crosses with the mutants above
should enable a conclusive ordering of markers; such a study was not
undertaken because it was considered peripheral to the main topic under
study.

Hist-1 was shown to be 15% linked to ile-1. Hist-1 was the terminal
marker investigated by Tyeryar et al., (1968), in their marker frequency
analysis of the B. licheniformis chromosome. These workers showed hist-1
to be 2% linked by transformation to a trp mutation. The position of
hist-1 with respect to ilyD1 has not been determined, though preliminary
studies with what is probably a hist-ily deletion, isolated after
hydroxylamine treatment of spores (Collins, personal communication),
indicates that at least one hist gene lies adjacent to ilyD1. The
putative \textit{met-ily} deletion does not revert to either \textit{met}^{+} or \textit{ily}^{+}, using \textit{NTG} as mutagen, the mutant has an absolute requirement for both methionine and isoleucine-valine and the two requirements have not been separated by recombination (<0.2\% recombination). It has not been possible to recombine \textit{ilyD1} and \textit{met-ily} (<0.2\% recombination). \textit{Met-ily} shows about 70\% linkage to \textit{iie-1} and 3-8\% linkage to \textit{pen}.

Because of the linkage of a \textit{thy} mutation to \textit{ilyD} and \textit{iie} mutations in \textit{B.subtilis} (Dubnau \textit{et al.}, 1967), a \textit{thy}^{−} mutant was isolated and the mutation (\textit{thy-1}) tested for linkage to \textit{ilyD1} and \textit{pen} in \textit{B.licheniformis}. A thymine requiring derivative of \textit{8a/1} was made by the procedure given in \textit{Materials and Methods}: 6b. Linkage of \textit{thy-1} to \textit{ilyD1}, \textit{pen} and \textit{ade-1} was tested in the following transformation experiments.

\begin{table}
\centering
\begin{tabular}{lll}
\textbf{Recipient}: & \textit{ST/1}; \textit{ade-1 thy-1 ilyD1 pen i^{p+}} & \textit{Donor}: 749/110\textit{penC3/22}.
\textbf{Transformant class} & \textbf{Transformants/ml.} & \textbf{Cotransfer frequency} \\
\textit{ade}^{+} & 11600 & \\
\textit{ade}^{+}\textit{pen i^{p}_{22}} & 39 & \text{3.4 x 10}^{-3} \\
\textit{ily}^{+} & 2516 & \\
\textit{ily}^{+}\textit{pen i^{p}_{22}} & 62 & \text{2.5 x 10}^{-2} \\
\textit{thy}^{+} & 12000 & \\
\textit{thy}^{+}\textit{pen i^{p}_{22}} & 168 & \text{1.4 x 10}^{-2} \\
*\textit{ily}^{+}\textit{thy}^{+} & 40 & \text{1.6 x 10}^{-2} \\
\end{tabular}
\caption{12a.}
\end{table}

DNA (about 0.2 \textmu g./ml.) was added immediately after dilution into TM for 60 min.

* Numbers of \textit{ily}^{+}\textit{thy}^{+} transformants were determined by replication of \textit{ily}^{+}\textit{thy}^{+} transformants onto plates lacking thymine.
As these results were suggestive of linkage between thy-1 and ilyD1/pen, a DNA dilution experiment was undertaken to test if the linkage was retained on DNA dilution. The results of the experiment are shown in Table 12b and Figure 3.

From Figure 3, it can be seen that the linkage between thy-1 and ilyD1 or pen is lost on DNA dilution, while that between ilyD1 and pen is retained. It is therefore concluded that thy-1 is not transformed on the same DNA molecule as ilyD1 and pen. However an appreciable cotransfer (2.3%) is obtained at DNA concentrations (6 x 10^{-2} μg./ml.) giving much lower cotransfer between other unlinked markers (e.g. 0.17% between ade-1 and pen).

The appreciable cotransfer that can be obtained between thy-1 and pen can be conveniently used for constructing strains carrying ade-1, ilyD1 and different pen mutations. ST recipients (i.e. ade-1 ilyD1 thy-1) are transformed with different pen mutants (phenotypically distinguishable from the recipient) and thy+ transformants selected. Penicillinase phenotypes are scored and the appropriate pen transformants (also ade-1 and ilyD1) are picked, purified and subsequently used in penicillinase mapping experiments.

In B. subtilis, two unlinked mutations are required to obtain a thy^- phenotype (Sicard and Anagnostopoulos, 1964; Anagnostopoulos and Schneider-Champagne, 1966; Wilson et al., 1965). One of these mutations (thyX) lies between ile and ilyD. Repair of either thyX or thyY (unlinked to thyX) gives a thy^+ phenotype.

If in B. licheniformis, the thy^- phenotype investigated above is due to two mutations, then neither of the two mutations lies between ilyD1 and ile-1, if repair of either of the mutations gives a thy^+ phenotype and both mutations can be repaired at a comparable frequency in transformation. Therefore until shown otherwise, the thy^- phenotype
Table 12b. Effect of DNA dilution on the linkage between thy-1, ade-1, ilvD1 and pen.

Recipient: ST/6; ade-1 ilvD1 thy-1 pen i322.   Donor: 9945A/WT

<table>
<thead>
<tr>
<th>DNA concentration (µg./ml.)</th>
<th>ade+</th>
<th>ade+ pen+</th>
<th>ade+ ilv+</th>
<th>thy+</th>
<th>thy+ pen+</th>
<th>thy+ ilv+</th>
<th>ilv+</th>
<th>ilv+ pen+</th>
<th>ilv+ thy+</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 x 10^-1</td>
<td>47900</td>
<td>495</td>
<td>467</td>
<td>43700</td>
<td>2038</td>
<td>152</td>
<td>6035</td>
<td>158</td>
<td>41</td>
</tr>
<tr>
<td>6 x 10^-2</td>
<td>18910</td>
<td>33</td>
<td>86</td>
<td>22310</td>
<td>500</td>
<td>38</td>
<td>964</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>6 x 10^-3</td>
<td>2598</td>
<td>1</td>
<td>3</td>
<td>2695</td>
<td>6</td>
<td>2</td>
<td>106</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6 x 10^-4</td>
<td>281</td>
<td>0</td>
<td>0</td>
<td>257</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Double auxotrophic transformants determined by replica plating.

\[ \text{pen}^+ = \text{i}^+ \text{p}^+ + \text{i}^+ \text{p}^22 \]

Recipient culture grown for 17 hours in BLSG, diluted 1/30 into TM containing DNA to the concentration indicated. Transformation stopped after 60 min. (40 µg. DNA'se for 15 min.)
Figure 3. Effect of DNA concentration on numbers of ade-1, thy-1
ilvD1, ade-1 thy-1, ilvD1 thy-1, ade-1 pen i_A^P^, thy-1 pen i_A^P^
and ilvD1 pen i_A^P^ transformants, using 8T/6 as recipient.
will be considered the result of a single mutation, \textit{thy-1}, which is unlinked to \textit{ilyD1}, \textit{ile-1} and \textit{pen}.

c) Conclusions

No markers more closely linked to \textit{pen} than \textit{ilyD1} and \textit{ile-1} have been found. Nevertheless a number of markers linked to \textit{ilyD1} and \textit{ile-1} have been obtained.

\textit{TrpE1} was generated after the use of nitrous acid treated DNA to repair \textit{ilyD1} in a recipient. \textit{TrpE1} is closely linked to other mutations blocking aromatic amino acid biosynthesis and is weakly linked to \textit{ilyD1}. These results indicate the similarity in genetic maps of \textit{B.licheniformis} and \textit{B. subtilis}. The finding that \textit{nat} mutations are linked to \textit{ilyD1} and \textit{ile-1}, confirms this similarity. Possible differences, other than the absence of a characterized penicillinase in \textit{B. subtilis}, are the absence of a \textit{thy} locus between \textit{ilyD} and \textit{ile} and the reversal of orientation of the aromatic gene cluster in the two organisms. These results, along with those of \textit{Tyeryar et al.}, have been compiled into a genetic map of \textit{B.licheniformis} (Appendix III).
B. GENETIC ANALYSIS OF Penicilllnase Mutations

1. Fine Structure Analysis

a) Introduction

The results from Section A show that the linkage between *ilyD1* or *ile-1*, and *pen* can be used to obtain large numbers of *pen* transformants at non-saturating DNA concentrations (<0.2 μg./ml.). *Pen* mutations can also be cotransformed (0.5-5%) with *thy-1* at concentrations approaching saturating. This linkage is lost on further DNA dilution.

In the mapping experiments described in this section, non-saturating DNA concentrations were used, though at some of the higher concentrations, the pseudo-linkage between *thy-1* and *pen* was used as well as that between *pen* and *ilyD1* or *ile-1*. Most of the penicillinase mutations were derived from strain 749 (Dubnau and Pollock, 1965). The initial experiments were to map structural mutations with respect to each other and orientate them with respect to mutations conferring magno-constitutivity. Other mutants of altered penicillinase phenotype were analysed genetically in the hope of locating and characterising the genetic blocks.

Mapping was by two-factor crosses, in which recombination values could be determined directly or indirectly. Three-factor crosses were used to verify marker orders and recombination values. Finally an experiment was undertaken to ascertain if penicillinase recombinants arising in this type of cross have resulted from the interaction of the recipient penicillinase genes with one or more transforming DNA molecules.

b) 2-Factor crosses

(1) Recombination between structural and regulatory gene mutations

The results of crosses between pairs of structural and regulatory
gene mutations are shown in Tables 13 and 14. Recombination values are
determined directly by comparison of donor and recombinant phenotype
frequencies. However the recombination values determined from a cross
and its reciprocal are not directly comparable.

e.g. for the reciprocal crosses:

\[
\begin{align*}
\text{(i)} & \\
\text{Recipient} & \quad \text{Donor} \\
+ & \quad + \\
\end{align*}
\]

Recombination function (R.F. (i))

\[
\frac{i_0^P +}{i_0^P + + i^P_1 +} 
\]

R.F. (i) = R.F. (ii) only when the same \(i_0^P +\) class of recombinants in (ii)
is equal in number to the \(i^P_1 +\) class of recombinants, which cannot be
scored.

Examination of the results in Tables 13 and 14, indicates that the
values of R.F. (i) and R.F. (ii) are comparable, as are the recombination
values in a cross, where different markers are used for selection.

The structural gene mutations \(p_{72}\) and \(p_{22}\) (Table 13) are the only
two penicillinase mutations investigated that produce no detectable
penicillinase activity (<0.05 U./mg.), though strains carrying either
of the mutations synthesize a CRM\(^+\) protein. The recombination data
suggest that \(p_{72}\) is closer to \(i_0\) than \(p_{22}\) is to \(i_{03}\).

In Table 14, the data for recombination between \(i_{03}\) and a number of
structural gene mutations are presented. The \(p_{26}\) mutation is classified
as structural on the basis of its abnormal reaction with antiserum
(Fleming, unpublished), though strains carrying this mutation are CRM\(^-\).
The recombination functions indicate that \(p_{26}\) and \(p_{16}\) are more distant
<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Cross</th>
<th>Selected marker</th>
<th>( \lambda^+ )</th>
<th>( \lambda^{+}\text{pen} )</th>
<th><strong>Recombination function</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>8T/7</td>
<td>9945A/WT</td>
<td>( i_0^p72 \times i^+p^+ )</td>
<td>ilv</td>
<td>4036</td>
<td>76 ( +p^+ )</td>
<td>8/64=0.095</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8 ( 0^p^+ )</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thy</td>
<td>5000</td>
<td>62 ( +p^+ )</td>
<td>10/72=0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10 ( 0^p^+ )</td>
<td></td>
</tr>
<tr>
<td>9T/1</td>
<td>749\text{pen}0/72</td>
<td>( i^+p^+ \times i_0^p72 )</td>
<td>ile</td>
<td>2751</td>
<td>64 ( p_72*** )</td>
<td>8/64=0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thy</td>
<td>2013</td>
<td>8 ( 0^p^+ )</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>75 ( p_72 )</td>
<td>5/75=0.067</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 ( 0^p^+ )</td>
<td></td>
</tr>
<tr>
<td>8T/6</td>
<td>9945A/WT</td>
<td>( i_0^p22 \times i^+p^+ )</td>
<td>ilv</td>
<td>7200</td>
<td>135 ( p_22 )</td>
<td>39/177=0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39 ( 0^p^+ )</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thy</td>
<td>9000</td>
<td>140 ( +p^+ )</td>
<td>30/170=0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 ( 0^p^+ )</td>
<td></td>
</tr>
<tr>
<td>9T/1</td>
<td>749/110\text{pen}03/22</td>
<td>( i^+p^+ \times i_0^p22 )</td>
<td>ile</td>
<td>7490</td>
<td>235 ( p_22 )</td>
<td>23/135=0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thy</td>
<td>2850</td>
<td>23 ( 0^p^+ )</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>34 ( p_22 )</td>
<td>5/34=0.15</td>
</tr>
</tbody>
</table>

* Recipient genotype first.
** See text.
*** \( p_72 = i^+p_72 + i_0^p_72 \) (i.e. \( \text{pen}^- \) phenotype).
Table 14. 2-Factor crosses to investigate recombination between a regulatory gene mutation ($i_{o3}$) and structural gene mutations. Recombination values calculated directly from the proportion of donor and recombinant phenotypes.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Cross</th>
<th><strong>Selected marker (X)</strong></th>
<th>Transforms/ml. $\Delta^+$</th>
<th>$\Delta^{men}$</th>
<th>Recombination function (1)</th>
<th>(ii)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8a/$i_{o3}^{p16}$</td>
<td>9945A/WT</td>
<td>$i_{o3}^{p16} \times i^{+}p^{+}$</td>
<td>ilv</td>
<td>1736</td>
<td>29 $i^{+}p^{+}$</td>
<td>6/34=0.15</td>
<td></td>
</tr>
<tr>
<td>81/1</td>
<td>749/110menC3/16</td>
<td>$i^{+}p^{+} \times i_{o3}^{p16}$</td>
<td>ilv</td>
<td>5110</td>
<td>65 $p_{16}^{+}$</td>
<td>6/65=0.092</td>
<td>11/79=0.14</td>
</tr>
<tr>
<td>8a/$i_{o3}^{p26}$</td>
<td>9945A/WT</td>
<td>$i_{o3}^{p26} \times i^{+}p^{+}$</td>
<td>ilv</td>
<td>3580</td>
<td>68 $i^{+}p^{+}$</td>
<td>11/79=0.14</td>
<td></td>
</tr>
<tr>
<td>81/1</td>
<td>749/110menC3/26</td>
<td>$i^{+}p^{+} \times i_{o3}^{p26}$</td>
<td>ilv</td>
<td>5855</td>
<td>72 $p_{26}$</td>
<td>13/72=0.18</td>
<td></td>
</tr>
<tr>
<td>81/1</td>
<td>749/110menC3/23</td>
<td>$i^{+}p^{+} \times i_{o3}^{p23}$</td>
<td>ilv</td>
<td>3117</td>
<td>45 $p_{23}$</td>
<td>5/45=0.11</td>
<td></td>
</tr>
</tbody>
</table>

*See text. **Recipient given first.

$\# p_{16} = i^{+}p_{16}^{+}$; $p_{23} = i^{+}p_{23}^{+}$; $p_{26} = i^{+}p_{26}^{+}$
than $p_{72}$ and $p_{23}$ to $i_{03}$. The enzyme synthesized by $749/110_{pen03/23}$ is thermodilabile and transformants containing $p_{23}$ were scored as low level penicillinase producers by the plate test, after the transformant plates had been incubated at $50^\circ C$ for 60 min.

(ii) Recombination between structural gene mutations

Reciprocal crosses between pairs of structural gene mutations would have involved constructing a large number of recipient strains carrying the structural gene mutations. Such a procedure is very time consuming and therefore an alternative procedure was adopted. The rationale was to cross structural gene mutants against recipients containing $pen^{-}p_{22}$ and $p_{72}^{-}$ such recipients are convenient to use because of their lack of penicillinase activity and because $p_{22}$ and $p_{72}$ are easily recombinable (15% recombination, Table 17). The data from such crosses should therefore allow a tentative ordering of markers.

The results from crosses between structural gene mutations, from which recombination values are calculated by a direct comparison of donor and recombinant phenotype frequencies, are shown in Table 15.

The highest recombination values (0.14) were between $p_{19}/p_{72}^{-}$ and between $p_{16}/p_{72}^{-}$, while for two pairs of mutations ($p_{19}/p_{22}^{-}$ and $p_{20}/p_{72}^{-}$) no recombinants were observed. In some of the crosses (crosses 2, 4 and 6) $pen^{-}i^{+}p^{+}$ recombinants could have been generated by recombination between $pen^{-}i_{C}$ and $i_{03}$. Few $pen^{-}i^{+}p^{+}$ recombinants were scored (less than 5% of $pen^{+}$ transformants) and they have been disregarded in Table 15. The low frequency of $i^{+}p^{+}$ recombinants indicates that $i_{C}$ and $i_{03}$ are very close together and/or more than two crossovers are required to generate such recombinants.

The number of 2-factor crosses in which recombination values could be determined directly was limited to penicillinase mutants which, when used as donors in transformation, had penicillinase levels phenotypically
Table 15. 2-Factor crosses between pairs of structural gene mutations. Recombination values determined directly.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Cross</th>
<th>Selected marker (X)</th>
<th>X+</th>
<th>X+men**</th>
<th>Recombination function</th>
<th>Mean**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 8T/6</td>
<td>749/110penC3/19</td>
<td>i_03^P_22 x i_03^P_19</td>
<td>ilv</td>
<td>1920</td>
<td>45 i_03^P_19</td>
<td>0/45 = &lt; 0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thy</td>
<td>3280</td>
<td>52 i_03^P_19</td>
<td>0/52 = &lt; 0.02</td>
<td></td>
</tr>
<tr>
<td>2. 8T/7</td>
<td>749/110penC3/19</td>
<td>i_03^P_72 x i_03^P_19</td>
<td>ilv</td>
<td>2160</td>
<td>40 i_03^P_19</td>
<td>5/45 = 0.11</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thy</td>
<td>2507</td>
<td>35 i_03^P_19</td>
<td>7/42 = 0.17</td>
<td></td>
</tr>
<tr>
<td>3. 8T/6</td>
<td>749/110penC3/16</td>
<td>i_03^P_22 x i_03^P_16</td>
<td>ilv</td>
<td>1480</td>
<td>33 i_03^P_16</td>
<td>2/35 = 0.06</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thy</td>
<td>2155</td>
<td>18 i_03^P_16</td>
<td>3/21 = 0.14</td>
<td>0.14</td>
</tr>
<tr>
<td>4. 8T/7</td>
<td>749/110penC3/16</td>
<td>i_03^P_72 x i_03^P_16</td>
<td>ilv</td>
<td>903</td>
<td>16 i_03^P_20</td>
<td>1/17 = 0.06</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thy</td>
<td>2119</td>
<td>20 i_03^P_20</td>
<td>3/23 = 0.13</td>
<td></td>
</tr>
</tbody>
</table>
Table 15 (cont).

<p>| | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>6. ST/7</td>
<td>749/110penC3/20</td>
<td>(i_{0}^{p}72\times i_{03}^{p}20)</td>
<td>ilv</td>
<td>534</td>
<td>8</td>
<td>(i_{p}^{20})</td>
<td>&lt;0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>thy</td>
<td>1164</td>
<td>0</td>
<td>(i_{p}^{+})</td>
<td>&lt;0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/8</td>
<td></td>
<td>0/14</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* Recipient given first.

** \(i^-\) is used where the \(i\) genotype is \(i_{03}\), \(i_{0}\) or \(i_{0}^{i_{03}}\).

*** Mean of values calculated for ilv and thy selection.
distinguishable from recipient and recombinant levels. With other mutants, indirect means had to be used for determining recombination values. The method used for these crosses was that of Nester and Lederberg (1961). A suitable recipient is transformed with a number of penicillinase mutant DNA's, having penicillinase levels indistinguishable from the recipient. The numbers of pen + recombinants (distinguishable from donor and recipient) resulting from such crosses are compared to the total number of pen + transformants arising from a cross using the same recipient and a donor of distinguishable penicillinase phenotype (i.e. reference cross).

The results from such crosses are shown in Tables 16a, 16b and 16c. In each case a given recipient is transformed with a number of DNA's (including one reference cross) under as similar conditions as possible, to eliminate the effect of variable linkage and differential appearance of transformants.

The results from these crosses give recombination values (recombination index, R.I.) comparable to those calculated directly.

A summary of recombination data for 2-factor crosses is given in Table 17.

c) 3-Factor crosses

The purpose of the 3-factor crosses was to verify the marker order suggested by the 2-factor crosses and to compare recombination values from these crosses with those obtained from the 2-factor crosses. 3-Factor crosses were limited to those for which suitable recipients had been constructed and tested in transformation; the results of such crosses are shown in Tables 18a, 18b and 18c.

Results of 3-factor reciprocal crosses are shown in Table 18a. The marker orders are unambiguous, though there is a higher frequency of recombinants generated by quadruple crossovers than expected. For
Table 16a. 2-Factor crosses in which donor pen phenotype cannot be distinguished phenotypically from that of the recipient. Recombination values between pairs of structural gene mutations determined by reference to total number of pen transformants from a reference cross. A single recipient culture transformed with the DNA’s shown, under identical conditions. Recipient: ST/7; ade-1 ilyD1 thy-1 penC/72

<table>
<thead>
<tr>
<th>Donor</th>
<th>Cross</th>
<th>Selected marker</th>
<th>Transformed/ml. X+</th>
<th>A&lt;sup&gt;+&lt;/sup&gt;</th>
<th>A&lt;sup&gt;+&lt;/sup&gt; pen&lt;sup&gt;+&lt;/sup&gt;</th>
<th>( \Lambda )</th>
<th>Recombination index (R.I.)&lt;sup&gt;**&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>9945A/WT</td>
<td>i&lt;sub&gt;o&lt;/sub&gt;p&lt;sub&gt;72&lt;/sub&gt; x i&lt;sup&gt;+&lt;/sup&gt;p</td>
<td>ilv</td>
<td>4036</td>
<td>76</td>
<td>i&lt;sup&gt;+&lt;/sup&gt;p</td>
<td>21</td>
<td>R.F. = 0.095</td>
</tr>
<tr>
<td>(reference)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>749/110pen&lt;sup&gt;03&lt;/sup&gt;/16</td>
<td>i&lt;sub&gt;o&lt;/sub&gt;p&lt;sub&gt;72&lt;/sub&gt; x i&lt;sub&gt;o&lt;/sub&gt;p&lt;sub&gt;16&lt;/sub&gt;</td>
<td>thy</td>
<td>2155</td>
<td>3</td>
<td>i&lt;sup&gt;-&lt;/sup&gt;p</td>
<td>1.4</td>
<td>R.F. = 0.097</td>
</tr>
<tr>
<td>749/110pen&lt;sup&gt;03&lt;/sup&gt;/26</td>
<td>i&lt;sub&gt;o&lt;/sub&gt;p&lt;sub&gt;72&lt;/sub&gt; x i&lt;sub&gt;o&lt;/sub&gt;p&lt;sub&gt;26&lt;/sub&gt;</td>
<td>thy</td>
<td>1833</td>
<td>8</td>
<td>i&lt;sup&gt;+&lt;/sup&gt;p</td>
<td>4.4</td>
<td>R.F. = 0.31</td>
</tr>
<tr>
<td>749/110pen&lt;sup&gt;03&lt;/sup&gt;/25</td>
<td>i&lt;sub&gt;o&lt;/sub&gt;p&lt;sub&gt;72&lt;/sub&gt; x i&lt;sub&gt;o&lt;/sub&gt;p&lt;sub&gt;25&lt;/sub&gt;</td>
<td>ilv</td>
<td>1530</td>
<td>4</td>
<td>i&lt;sup&gt;-&lt;/sup&gt;p</td>
<td>2.6</td>
<td>R.F. = 0.12</td>
</tr>
<tr>
<td>749/110pen&lt;sup&gt;03&lt;/sup&gt;/19</td>
<td>i&lt;sub&gt;o&lt;/sub&gt;p&lt;sub&gt;72&lt;/sub&gt; x i&lt;sub&gt;o&lt;/sub&gt;p&lt;sub&gt;19&lt;/sub&gt;</td>
<td>thy</td>
<td>2160</td>
<td>5</td>
<td>i&lt;sup&gt;-&lt;/sup&gt;p</td>
<td>2.3</td>
<td>R.F. = 0.19</td>
</tr>
</tbody>
</table>

\[
\Lambda = \frac{A^{+} \text{pen}^{+}}{A^{+}} \times 10^3
\]

* R.I. = \( \frac{\Lambda \text{(mutant)}}{\Lambda \text{(reference)}} \)

** R.F. = Recombination function
<table>
<thead>
<tr>
<th>Donor</th>
<th>Cross</th>
<th>Selected marker (X)</th>
<th>X+ Transformants/μl.</th>
<th>X+pen+</th>
<th>A*</th>
<th>R.I. **</th>
</tr>
</thead>
<tbody>
<tr>
<td>749/110 penC3/19 (reference)</td>
<td>i&lt;sub&gt;0&lt;/sub&gt;C&lt;sub&gt;3&lt;/sub&gt;P&lt;sub&gt;22&lt;/sub&gt; x i&lt;sub&gt;0&lt;/sub&gt;C&lt;sub&gt;3&lt;/sub&gt;P&lt;sub&gt;19&lt;/sub&gt;</td>
<td>ilv</td>
<td>2632</td>
<td>64</td>
<td>24</td>
<td>reference</td>
</tr>
<tr>
<td></td>
<td></td>
<td>thy</td>
<td>2183</td>
<td>71</td>
<td>32</td>
<td>reference</td>
</tr>
<tr>
<td>749 penC/72</td>
<td>i&lt;sub&gt;0&lt;/sub&gt;C&lt;sub&gt;3&lt;/sub&gt;P&lt;sub&gt;22&lt;/sub&gt; x i&lt;sub&gt;0&lt;/sub&gt;C&lt;sub&gt;72&lt;/sub&gt;</td>
<td>ilv</td>
<td>3000</td>
<td>10</td>
<td>3.3</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>thy</td>
<td>3897</td>
<td>13</td>
<td>3.3</td>
<td>0.10</td>
</tr>
<tr>
<td>749/110 penC3/16</td>
<td>i&lt;sub&gt;0&lt;/sub&gt;C&lt;sub&gt;3&lt;/sub&gt;P&lt;sub&gt;22&lt;/sub&gt; x i&lt;sub&gt;0&lt;/sub&gt;C&lt;sub&gt;3&lt;/sub&gt;P&lt;sub&gt;16&lt;/sub&gt;</td>
<td>thy</td>
<td>5480</td>
<td>3</td>
<td>0.55</td>
<td>0.017</td>
</tr>
<tr>
<td>749/110 penC3/25</td>
<td>i&lt;sub&gt;0&lt;/sub&gt;C&lt;sub&gt;3&lt;/sub&gt;P&lt;sub&gt;22&lt;/sub&gt; x i&lt;sub&gt;0&lt;/sub&gt;C&lt;sub&gt;3&lt;/sub&gt;P&lt;sub&gt;25&lt;/sub&gt;</td>
<td>ilv</td>
<td>6552</td>
<td>8</td>
<td>1.3</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td></td>
<td>thy</td>
<td>8590</td>
<td>8</td>
<td>0.94</td>
<td>0.039</td>
</tr>
<tr>
<td>749/110 penC3/26</td>
<td>i&lt;sub&gt;0&lt;/sub&gt;C&lt;sub&gt;3&lt;/sub&gt;P&lt;sub&gt;22&lt;/sub&gt; x i&lt;sub&gt;0&lt;/sub&gt;C&lt;sub&gt;3&lt;/sub&gt;P&lt;sub&gt;26&lt;/sub&gt;</td>
<td>ilv</td>
<td>10720</td>
<td>4</td>
<td>0.37</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>thy</td>
<td>14480</td>
<td>7</td>
<td>0.48</td>
<td>0.015</td>
</tr>
</tbody>
</table>

\[
\%A = \frac{A^{+\text{pen+}}}{A^{+}} \times 10^3 \quad \text{**R.I.} = \frac{A(\text{mutant})}{A(\text{reference})}
\]
Table 16c. 2-Factor crosses to determine recombination index (R.I.) between pairs of structural gene mutations.

**Recipient:** ST/7; ade-1 ilvD1 thy-1 pen i0p72

<table>
<thead>
<tr>
<th>Donor</th>
<th>Cross</th>
<th>Selected marker (X)</th>
<th>Transforms/ml.</th>
<th>$\frac{X^+_{pen^+}}{X^+}$</th>
<th>$\frac{X^+_{pen^+}}{X^+} \times 10^3$</th>
<th>R.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>749/110pen28</td>
<td>$i_0^p72 \times r_28^p$</td>
<td>ilv</td>
<td>282</td>
<td>7 7</td>
<td>24.8</td>
<td>reference</td>
</tr>
<tr>
<td>(reference)</td>
<td></td>
<td></td>
<td></td>
<td>0 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10 r_{28}^p</td>
<td>15.5</td>
<td>reference</td>
</tr>
<tr>
<td>749/110pen03/22</td>
<td>$i_0^p72 \times i_0^3p22$</td>
<td>thy</td>
<td>4748</td>
<td>10 i_{p}^-</td>
<td>2.1</td>
<td>0.14</td>
</tr>
<tr>
<td>749/110pen03/25</td>
<td>$i_0^p72 \times i_0^3p25$</td>
<td>thy</td>
<td>15200</td>
<td>25 i_{p}^-</td>
<td>1.6</td>
<td>0.10</td>
</tr>
<tr>
<td>749/110pen03/26</td>
<td>$i_0^p72 \times i_0^3p26$</td>
<td>thy</td>
<td>2078</td>
<td>4 i_{p}^-</td>
<td>1.9</td>
<td>0.12</td>
</tr>
</tbody>
</table>

R.I. = \frac{\frac{X^+_{pen^+}}{X^+}}{\frac{X^+_{pen^+}}{X^+} \text{ mutant}} \frac{X^+_{pen^+}}{X^+} \text{ reference}
Table 17. A summary of recombination data deduced from the results of 2-factor crosses.

<table>
<thead>
<tr>
<th>Recombination between</th>
<th>R.F.(i)</th>
<th>*Recombination</th>
<th>R.F.(ii)</th>
<th>R.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>i_0/p_72</td>
<td>0.12</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i_0/p_22</td>
<td>0.20</td>
<td>0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i_0/p_16</td>
<td>0.15</td>
<td>0.092</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i_0/p_26</td>
<td>0.14</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i_0/p_23</td>
<td></td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p_22/p_19</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p_22/p_16</td>
<td>0.06</td>
<td>0.017</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p_22/p_20</td>
<td>0.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p_22/p_25</td>
<td></td>
<td>0.047</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p_22/p_26</td>
<td></td>
<td>0.015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p_72/p_19</td>
<td>0.14</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p_72/p_16</td>
<td>0.14</td>
<td>0.097</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p_72/p_20</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p_72/p_25</td>
<td></td>
<td>0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p_72/p_26</td>
<td></td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p_72/p_22</td>
<td></td>
<td>0.13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Each recombination value is the mean of the values calculated from a given cross, where different markers are selected (i.e. ilvD1, ile-1 or thy-1).
Table 18a. 3-Factor reciprocal crosses to determine marker order.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>*Cross</th>
<th>Selected marker (X)</th>
<th>Transforms/ml.</th>
<th>Suggested order</th>
<th>Recombination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 9b/2</td>
<td>749/110pen03/22</td>
<td>i\textsuperscript{+}p\textsubscript{72} x i\textsubscript{03}p\textsubscript{22}</td>
<td>ile</td>
<td>10670</td>
<td>7 \text{i\textsubscript{03}}p\textsuperscript{+} \text{i\textsubscript{p}}</td>
<td>i\textsubscript{03}/p\textsubscript{72} = 0.13</td>
</tr>
<tr>
<td>2. ST/6</td>
<td>9b/2</td>
<td>i\textsubscript{03}p\textsubscript{22} x i\textsuperscript{+}p\textsubscript{72}</td>
<td>ilv</td>
<td>4500</td>
<td>4 i\textsubscript{p} \text{i\textsuperscript{+}}</td>
<td>i\textsubscript{03}p\textsubscript{72}p\textsubscript{22}</td>
</tr>
<tr>
<td>3. 9b/2</td>
<td>749/110pen03/26</td>
<td>i\textsuperscript{+}p\textsubscript{72} x i\textsubscript{03}p\textsubscript{26}</td>
<td>ile</td>
<td>6900</td>
<td>9 \text{i\textsubscript{03}}p\textsuperscript{+}</td>
<td>i\textsubscript{03}/p\textsubscript{72} = 0.25</td>
</tr>
<tr>
<td>4. 8a/i\textsubscript{03}p\textsubscript{26}</td>
<td>9b/2</td>
<td>i\textsubscript{03}p\textsubscript{26} x i\textsuperscript{+}p\textsubscript{72}</td>
<td>ilv</td>
<td>5120</td>
<td>5 \text{i\superscript{+}}p \text{i\textsubscript{03}}p\textsuperscript{+} \text{i\textsubscript{p}}</td>
<td>i\textsubscript{03}p\textsubscript{72}p\textsubscript{26}</td>
</tr>
<tr>
<td>5. 8a/i\textsuperscript{+}p\textsubscript{26}</td>
<td>749pen072</td>
<td>i\textsuperscript{+}p\textsubscript{26} x i\textsubscript{03}p\textsubscript{72}</td>
<td>ilv</td>
<td>6900</td>
<td>4 \text{i\textsubscript{03}}p\textsuperscript{+}</td>
<td>i\textsubscript{03}/p\textsubscript{72}p\textsubscript{26}</td>
</tr>
<tr>
<td>6. ST/7</td>
<td>9945A ade-1 pen</td>
<td>i\textsuperscript{+}p\textsubscript{26} i\textsubscript{03}p\textsubscript{72} x i\textsuperscript{+}p\textsubscript{26}</td>
<td>ilv</td>
<td>3467</td>
<td>3 \text{i\superscript{+}}p \text{i\textsubscript{03}}p\textsuperscript{+} \text{i\textsubscript{p}}</td>
<td>i\textsubscript{03}/p\textsubscript{72} &lt; 0.25</td>
</tr>
</tbody>
</table>

* Recipient given first.
Table 16b. 3-Factor crosses to determine marker order and verify recombination frequencies between structural gene mutations. Results compiled from two transformation experiments, using the same recipient.

**Recipient**: 9b/2; *ile-1 pen i⁺<sub>p72</sub>

<table>
<thead>
<tr>
<th>Donor</th>
<th>Cross</th>
<th>Selected marker (X)</th>
<th>Transforms/ml.</th>
<th>Suggested order</th>
<th>Recombination function</th>
</tr>
</thead>
<tbody>
<tr>
<td>749/110&lt;sub&gt;men&lt;/sub&gt;03/25</td>
<td>i⁺&lt;sub&gt;p72&lt;/sub&gt; x i₀&lt;sub&gt;3&lt;/sub&gt;p&lt;sub&gt;25&lt;/sub&gt;</td>
<td>ile</td>
<td>8000</td>
<td>11 i₀&lt;sub&gt;3&lt;/sub&gt;p⁺</td>
<td>i₀&lt;sub&gt;3&lt;/sub&gt;-p&lt;sub&gt;72&lt;/sub&gt;-p&lt;sub&gt;25&lt;/sub&gt;</td>
</tr>
<tr>
<td>749/110&lt;sub&gt;men&lt;/sub&gt;03/16</td>
<td>i⁺&lt;sub&gt;p72&lt;/sub&gt; x i₀&lt;sub&gt;3&lt;/sub&gt;p&lt;sub&gt;16&lt;/sub&gt;</td>
<td>ile</td>
<td>3002</td>
<td>2 i₀&lt;sub&gt;3&lt;/sub&gt;p⁺</td>
<td>i₀&lt;sub&gt;3&lt;/sub&gt;-p&lt;sub&gt;72&lt;/sub&gt;-p&lt;sub&gt;16&lt;/sub&gt;</td>
</tr>
<tr>
<td>749/110&lt;sub&gt;men&lt;/sub&gt;03/23</td>
<td>i⁺&lt;sub&gt;p72&lt;/sub&gt; x i₀&lt;sub&gt;3&lt;/sub&gt;p&lt;sub&gt;23&lt;/sub&gt;</td>
<td>ile</td>
<td>4110</td>
<td>3 i₀&lt;sub&gt;3&lt;/sub&gt;p⁺</td>
<td>i₀&lt;sub&gt;3&lt;/sub&gt;-p&lt;sub&gt;72&lt;/sub&gt;-p&lt;sub&gt;23&lt;/sub&gt;</td>
</tr>
<tr>
<td>749&lt;sub&gt;men&lt;/sub&gt;0/71</td>
<td>i⁺&lt;sub&gt;p72&lt;/sub&gt; x i₀&lt;sub&gt;3&lt;/sub&gt;p&lt;sub&gt;71&lt;/sub&gt;</td>
<td>ile</td>
<td>3627</td>
<td>0 i⁺&lt;sub&gt;p&lt;/sub&gt;</td>
<td>-</td>
</tr>
<tr>
<td>749/110&lt;sub&gt;men&lt;/sub&gt;03/25</td>
<td>i⁺&lt;sub&gt;p72&lt;/sub&gt; x i₀&lt;sub&gt;3&lt;/sub&gt;p&lt;sub&gt;25&lt;/sub&gt;</td>
<td>ile</td>
<td>9600</td>
<td>26 i₀&lt;sub&gt;3&lt;/sub&gt;p⁺</td>
<td>i₀&lt;sub&gt;3&lt;/sub&gt;-p&lt;sub&gt;72&lt;/sub&gt;-p&lt;sub&gt;25&lt;/sub&gt;</td>
</tr>
<tr>
<td>749/110&lt;sub&gt;men&lt;/sub&gt;03/16</td>
<td>i⁺&lt;sub&gt;p72&lt;/sub&gt; x i₀&lt;sub&gt;3&lt;/sub&gt;p&lt;sub&gt;16&lt;/sub&gt;</td>
<td>ile</td>
<td>4515</td>
<td>4 i₀&lt;sub&gt;3&lt;/sub&gt;p⁺</td>
<td>i₀&lt;sub&gt;3&lt;/sub&gt;-p&lt;sub&gt;72&lt;/sub&gt;-p&lt;sub&gt;16&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

* Second transformation experiment.
Table 16c. 3-Factor crosses to determine marker order.

**Recipient**: 8a/i<sup>+</sup><sub>p26</sub>; ade-1 ilyD1 pen i<sup>+</sup><sub>p26</sub>

<table>
<thead>
<tr>
<th>Donor</th>
<th>Cross</th>
<th>Transforms/ml.</th>
<th>Suggested order</th>
<th>Recombination function</th>
</tr>
</thead>
<tbody>
<tr>
<td>749/110penC3/22</td>
<td>i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;p26&lt;/sub&gt; x i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;03&lt;/sub&gt;&lt;sup&gt;p22&lt;/sup&gt;</td>
<td>11530</td>
<td>6 i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;03&lt;/sub&gt;</td>
<td>i&lt;sub&gt;03&lt;/sub&gt;-&lt;sup&gt;p26&lt;/sup&gt;-&lt;sub&gt;p22&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;i&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>749/110penC3/25</td>
<td>i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;p26&lt;/sub&gt; x i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;03&lt;/sub&gt;&lt;sup&gt;p25&lt;/sup&gt;</td>
<td>6440</td>
<td>11 i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;i&lt;/sub&gt;</td>
<td>i&lt;sub&gt;03&lt;/sub&gt;-&lt;sup&gt;p25&lt;/sup&gt;-&lt;sub&gt;p26&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;03&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>115 i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;03&lt;/sub&gt;&lt;sup&gt;p25&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>749/110penC3/17</td>
<td>i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;p26&lt;/sub&gt; x i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;03&lt;/sub&gt;&lt;sup&gt;p17&lt;/sup&gt;</td>
<td>9990</td>
<td>3 i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;03&lt;/sub&gt;</td>
<td>i&lt;sub&gt;03&lt;/sub&gt;-&lt;sup&gt;p26&lt;/sup&gt;-&lt;sub&gt;p17&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>189 i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;03&lt;/sub&gt;&lt;sup&gt;p17&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2 ml.)</td>
<td></td>
<td>0 i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;i&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>749penC/72</td>
<td>i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;p26&lt;/sub&gt; x i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;072&lt;/sub&gt;</td>
<td>13550</td>
<td>25 i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;i&lt;/sub&gt;</td>
<td>i&lt;sub&gt;072&lt;/sub&gt;-&lt;sub&gt;p26&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 i&lt;sup&gt;+&lt;/sup&gt;&lt;sub&gt;03&lt;/sub&gt;</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4. Genetic map of the penicillinase i and p genes. Compiled from the results of 2- and 3-factor crosses (Tables 1-21). The recombination values given are the mean of those calculated from different crosses and after selection for different markers. The relative orders of most of the markers was deduced from the results from 3-factor crosses (Tables 18a, 19b, 18c, 19 and 21). A single arrow head indicates the donor. Double arrow heads indicate that either both mutations were in the same strain, or that the recombination value given is the mean of those calculated from a cross and its reciprocal.

Pen p₁₉ has not been recombined with p₂₂ (less than 0.5% recombination) and p₇₁ and p₂₀ have not been recombined with p₇₂ (less than 2% recombination).
example, in crosses 2, 4 and 5 (Table 18a), a quadruple crossover is required to generate either $\text{pen} i^p+$ (crosses 2 and 4) or $i_p^+$ (cross 5) recombinants. The frequency of these recombinant classes is as high as that for the generation of the same class of recombinants by a double crossover in the reciprocal cross. This same behaviour can be seen from the data in Tables 18b and 18c. No explanation of this behaviour can be given. Despite this high frequency of quadruple crossovers, the suggested orders from reciprocal crosses are unambiguous and for other 3-factor crosses, the orders suggested are consistent with the recombination values calculated from the results of 2-factor crosses. The data from the 2- and 3-factor crosses have been compiled into a map of the penicillinase region (Figure 4).

d) Analysis of mutations conferring macro-constitutivity (i)

A number of $\text{i}$ mutations, including a temperature-sensitive $\text{i}$ mutant (749penT9), were analysed. Mapping $\text{i}$ mutants with respect to each other by 2-factor crosses is not practicable; the high penicillinase levels produced by the majority of colonies decolorize plates very quickly using the penicillin/iodine plate test and inducible recombinants are missed. However with 3-factor crosses, in which the recipient carries a structural gene mutation as well as an $\text{i}$ mutation (i.e. pen$^-$ phenotype), the numbers of $i_p^+$ and $i^+_p$ transformants were easily scored against the pen$^-$ recipient background.

The results from such crosses are shown in Table 19. The data is unambiguous and suggests the order:—

\[
\text{i}_9 \quad \text{i}_9 \quad \text{i}_9 \quad \text{STRUCTURAL GENE}
\]

Reciprocal crosses using pen $i_9$ in a recipient were not undertaken because the necessary strains have not been constructed.

The data in Table 19 also allow the calculation of the following
<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Cross</th>
<th>Transformants/ml.</th>
<th>Suggested order</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 8a/6</td>
<td>749pen0</td>
<td>(i_0^+ \times i_0^+_{p22})</td>
<td>3945 81 (i^-_p)</td>
<td>10 (i_0^+)</td>
</tr>
<tr>
<td>2, 8a/7</td>
<td>749/110pen03</td>
<td>(i_0^+<em>{p72} \times i_0^+</em>{p72})</td>
<td>7120 210 (i^-_p)</td>
<td>18 (i^+_p)</td>
</tr>
<tr>
<td>3, 8a/6</td>
<td>749penT9</td>
<td>(i_{T9}^+ \times i_0^+_{p22})</td>
<td>**3230 80 (i_{T9}^+)</td>
<td>**10 (i_{T9}^+)</td>
</tr>
<tr>
<td>4, 8a/7</td>
<td>749penT9</td>
<td>(i_{T9}^+ \times i_0^+_{p72})</td>
<td>**5390 140 (i_{T9}^+)</td>
<td>**9 (i_{T9}^+)</td>
</tr>
</tbody>
</table>

* Donor genotype given first.

** Transformants grown at 37°C, at which temperature \(i_{T9}^+\) transformants have the same phenotype as \(i^+_p\) colonies and can be distinguished from \(i_0^+\) or \(i_0^+_{p72}\) transformants. Colonies of inducible-like phenotype picked off and grown at 47°C, when \(i_{T9}^+\) transformants are distinguishable from \(i^+_p\) transformants.

* These will presumably include double \(i\) mutation transformants, i.e. \(i_0^+i_{T9}^+\) and \(i_0^+i_{T9}^+\), unless the two mutations complement each other.
recombination values:

\[
\begin{align*}
&i_{C}/p_{72} = 0.057; \quad i_{T9}/p_{72} = 0.11; \quad i_{T9}/i_{0} = 0.051 \text{ (cross 4)} \\
&i_{C}/p_{22} = 0.11 \text{ (cross 3)} \\
&i_{03}/i_{C} = 0.08 \text{ (cross 2), assuming the value of 0.057 for } i_{C}/p_{72} \text{ (cross 4)}
\end{align*}
\]

e) DNA competition experiment

The purpose of this experiment was to determine if under the conditions used for the mapping experiments, pen recombinants have resulted from the interaction of the penicillinase region of the chromosome with one or more transforming DNA molecules.

<table>
<thead>
<tr>
<th>Table 20.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient: 9b/2; \text{ ile-1 pen i}^{+}_{p72}</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Donor DNA</th>
<th>Cross</th>
<th>Transformants/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>*Concentration</td>
<td>\text{i}^{+}<em>{p72} \times \text{i}^{-}</em>{p72}</td>
</tr>
<tr>
<td>749\text{penC}/72</td>
<td>0.2</td>
<td>23100</td>
</tr>
<tr>
<td>9945A/WT</td>
<td>0.2</td>
<td>25400</td>
</tr>
<tr>
<td>749\text{penC}/72 + 9945A/WT</td>
<td>0.2</td>
<td>39600</td>
</tr>
</tbody>
</table>

\* Approximate concentrations (ug./ml.).

It can therefore be concluded that the majority of penicillinase transformants have resulted from the interaction of a single transforming DNA molecule with the penicillinase region of the recipient. Since recombination frequencies for the penicillinase region seem to be independent of the marker selected it can be assumed that this conclusion is valid for all the penicillinase mapping experiments presented, whatever the marker selected.
Conclusions

Fine structure mapping of the penicillinae structural gene (p) and a closely linked gene controlling inducibility (i) has been possible using 2- and 3-factor crosses. The results of the crosses have allowed most mutations to be placed in an unambiguous order (Figure 4). Recombination values are reasonably additive and the recombination values obtained from a cross and its reciprocal are usually comparable. In three-factor crosses a higher frequency of quadruple crossovers than theory predicts was observed.

All of the penicillinae structural gene mutations except p25, p26, and p17 were originally classified as structural gene mutations, because strains carrying these mutations are CRH+, and produce a penicillinase of lowered specific activity and react abnormally with antiserum. Strains carrying p25, p26, and p17 are CRH- and were originally classified as regulatory gene mutations (Dubnau and Pollock, 1965; Fleming, unpublished). 749/110pen03/26 produces a penicillinase whose reaction with antiserum is slightly abnormal (Fleming, unpublished). 749/110pen03/25 and 749/110pen03/17 produce normal penicillinase judged by their reaction with antiserum. The mapping data suggest that all these mutations lie in the penicillinae structural gene. Mutations lying in the penicillinae structural gene and producing small amounts of normal or near-normal penicillinase could be nonsense mutations which are being suppressed to a low level. The nature of these mutations is discussed further in the Discussion.

It is interesting to note that the two outside markers in the structural gene (p22 and p72) are the only two penicillinae mutations investigated that produce no detectable penicillinae activity. This and the fact that other mutations producing significant penicillinae activity do not recombine with p22 and p72 (p19 does not recombine with
$p_{22}$ (<0.5\% recombination), $p_{71}$ and $p_{20}$ have not been recombined with $p_{72}$ (<2\% recombination)) suggests that $p_{22}$ and $p_{72}$ may be deletions or close multi-site mutations. NTG, the mutagen used to generate most of the penicillinase mutations is known to give multi-site mutations and deletions at a high frequency.

2. **AN EXTERNALLY SUPPRESSED PENICILLINASE MUTATION**

749/110penC3/17 was derived from 749/110penG3 by NTG (Dubnau and Pollock, 1965) and produces 20 U./mg. dry wt. bacteria of penicillinase and is uninducible. The enzyme synthesized reacts normally with wild-type antiserum and has a normal substrate profile (J. Fleming, unpublished). 749/110penC3/17 colonies are CRM when tested against wild-type antiserum in a surface growth Ouchterlony test (Pollock, 1964). This evidence suggests that the strain produces low amounts of normal or near-normal penicillinase. Mapping experiments were undertaken to locate the mutation or mutations responsible for the observed penicillinase phenotype.

The results of the initial experiments are shown in Table 21. Most of the pen$^+$ transformants produced only low levels of penicillinase (<5 U./mg.) judged by the plate test. When these colonies were picked, purified and assayed in liquid culture, they were found to synthesize 2–4 U./mg. of penicillinase and to be uninducible. Only 6/350 of the pen$^+$ transformants tested had the donor penicillinase phenotype; pen $^{i+}p^+$ and pen $^{i0}p^+$ recombinants were also produced.

These results suggest that the penicillinase level of 749/110penC3/17 is due to two mutations; one in the penicillinase region, giving rise to an activity of 2–4 U./mg., and the other at an unlinked locus (suc-1), which increases the level to 20 U./mg. of penicillinase. During transformation the inheritance of the unlinked locus is a rare event (6/350).
at non-saturating DNA concentrations. The recombination data suggest that the mutation in the penicillinase region ($p_{17}$) is close to $p_{22}$ and $p_{26}$.

Table 21. Initial mapping experiments with strain 749/110pen03/17.

**Donor:** 749/110pen03/17

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Cross</th>
<th>Transformants/ml.</th>
<th>ilv+</th>
<th>ilv*pen+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 8a/i$^+$p_{26} \times i_{03}p_{17}</td>
<td>9990 185 pen+ (low: &lt;5 U./mg. and uninducible) (2ml.)</td>
<td>4 pen+ (medium: about 20 U./mg. and uninducible)</td>
<td>3 i_{03}p+</td>
<td></td>
</tr>
<tr>
<td>2. 8a/6 \times i_{03}p_{22} x i_{03}p_{17}</td>
<td>1224 22 pen+ (low: &lt;5 U./mg., uninducible)</td>
<td>3 pen+ (medium: about 20 U./mg. 2/4 inducible)</td>
<td>15 i_{03}p+</td>
<td></td>
</tr>
<tr>
<td>3. 9b/2 \times i_{03}p_{72} x i_{03}p_{17}</td>
<td>5620 135 pen+ (low: &lt;5 U./mg. and uninducible) (ilet+)</td>
<td>4 pen+ (medium: about 20 U./mg.</td>
<td>13 i_{03}p+</td>
<td></td>
</tr>
</tbody>
</table>

Recombination values are: $p_{26}/p_{17} = 3/192 = 0.016$ $p_{72}/p_{17} = 13/150 = 0.087$ $p_{22}/p_{17} = <0.048$

These values assume that the $pen^+$ transformants producing 2–4 U./mg. penicillinase have the genotype $i_{03}p_{17}$ $sup^{-1}$ and the transformants with donor-type activity have the genotype $i_{03}p_{17}$ $sup^{-1}$. Any $pen^+ i_{p_{17}}$ recombinants would be expected to produce very little penicillinase and would not be scored as $pen^+$.

In the 3-factor crosses: $i_{p_{26}} x i_{03}p_{17}$ and $i_{p_{72}} x i_{03}p_{17}$ (Table 21), the predominance of $i_{03}p^+$ recombinants over $i_{p}^+$, suggests that the $p_{17}$ mutation lies close to $p_{26}$ and $p_{22}$, on the $p_{72}$ distal side of $p_{26}$. The...
lack of an $i^{+}\text{p}_{22}$ strain prevented ordering with respect to $p^{+}_{22}$, though the proximity of $p_{17}$ to $p_{26}$ suggests that the mutation lies between $p_{26}$ and $p_{22}$, i.e. within the structural gene.

\[
\begin{array}{cccc}
\text{p}_{72} & \text{p}_{26} & \text{p}_{17} & \text{p}_{22} \\
0.087 & 0.016 & \text{<0.048} \\
\end{array}
\]

A possible explanation of the behaviour of 749/110\text{pen}03/17 in these crosses is that the unlinked mutation is a suppressor mutation responsible for increasing the level of $p_{17}$. The properties of $p_{17}$ suggest that it could be a nonsense mutation, and in the absence of the suppressor mutation, the penicillinase activity of 2-4 U./mg. results from some slight natural suppression or misreading.

Strain 749/110\text{pen}03/17 carries a streptomycin resistance mutation ($\text{str}^{-1}$), while recipients used for transformation are $\text{str}^{-1+}$ (streptomycin sensitive). In E. coli, mutations giving rise to altered 30S ribosomal sub-units are known to affect translational misreading; a ribosomal ambiguity mutation ($\text{arb}$) in a $\text{str}^{-}$ (streptomycin sensitive) strain can suppress all three nonsense mutations (Rosset and Gorini, 1969). Mutations at the $\text{str}^{-}$ locus, giving rise to streptomycin resistance, lower the suppression efficiency of both amber and ochre suppressors (Gorini and Beckwith, 1966) and suppression by $\text{arb}$. Other mutations in E. coli, resulting in altered ribosomes, are able to suppress unlinked mutations (Apirion, 1966; Gartner et al., 1969). It therefore seemed possible that the suppression in strain 749/110\text{pen}03/17 was due to the presence of the $\text{str}^{-1}$ mutation.

Two \text{illy}^{+} transformants having donor penicillinase phenotypes (Table 21, cross 1) and presumably having the genotype 9945A $\text{ade}^{-1}$ $\text{pen}$
were both found to be $\text{str}^R(\text{str-1})$. Penicillinase transformants producing 2-4 U./mg. penicillinase were $\text{str}^S$ (7 tested). These results suggest that the $\text{str-1}$ mutation, or a mutation closely linked, is responsible for the suppression. To test this possibility, a suitable recipient was constructed with the $\text{str-1}$ allele in the following transformation experiment.

Recipient: 6a/7; ade-1 ilvD1 pen $i_0^{-3}$. 72
Donor: 749/110pen03/17 ($\text{str-1}$)

After transformation, using a saturating DNA concentration (2 μg./ml. for 2 hours), $\text{str}^R$ transformants were selected. Of the 26 pen$^+$str$^R$ transformants scored, 2 were pen$^+$ and 26 produced 20 U./mg. of penicillinase and were uninducible. No transformants producing 2-4 U./mg. of penicillinase were found. Another similar experiment produced 129 str-1 pen$^+$ transformants, of which none produced the unsuppressed level of penicillinase (2-4 U./mg.). Therefore, if the suppressing mutation is at a distinct site to str-1, there is less than 0.7% recombination between the two mutant sites. One of the str$^R$ pen$^-$ transformants from the transformation above was now used as a recipient to verify the suppressing activity of the str-1 locus, (see Table 22).

It is concluded that the unlinked suppressor locus (sup-1) can increase the penicillinase level in a strain carrying $i_0^{-3}$. 17 from 2-4 U./mg. to 20 U./mg. penicillinase. The sup-1 locus is probably identical to str-1.

The str-1 mutation in strain 749/110pen03/17 was originally generated by Dubnau and Pollock (1965) in strain 749/110 (his-1 str-1 pen $i_0^+$. $p^+$), which is the parent strain of many of the penicillinase mutants used in this work. It was therefore possible to test whether
Table 22.

Recipient: 9945A ade-1 ilvD1 str-1 pen i03p72

Donor: 749/110penC3/17 (str-1)
9945A ade-1 str-1+ pen i03p17

<table>
<thead>
<tr>
<th>Donor DNA</th>
<th>Transforms/0.6ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ilv+</td>
</tr>
<tr>
<td>749/110penC3/17 str-1</td>
<td>894</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>9945A ade-1 str-1+ pen i03p17</td>
<td>645</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All ilv+ pen+ transforms were strR.
the original str-1 allele had suppressor activity, or whether the suppressor had been introduced during the mutagenesis of 749/110, leading to the isolation of 749/110\textsubscript{\textit{men} 03/17}.

A strain derived from 749/110, having a distinguishable penicillinase phenotype was chosen as donor and used to transform an \textit{ilv}D\textsubscript{1} \textit{pen} \textit{icp}_{17} str-1\textsuperscript{+} recipient. The donor was 749/110\textit{men}31 (str-1), which produces 9 U./mg. of penicillinase and is uninducible (Dubnau and Pollock, 1965; Fleming, unpublished). The recipient, producing 2-4 U./mg. of penicillinase, was transformed with 749/110\textit{men}31 DNA (about 0.2 µg. for 60 min.) and str\textsuperscript{R} transforms were selected. More than 99% of str\textsuperscript{R} colonies produced about 20 U./mg. penicillinase. Pen\textsubscript{31} and recombinant-type (pen \textit{icp}\textsuperscript{+}) levels of penicillinase were also observed (≤ 5%). No colonies producing 2-4 U./mg. of penicillinase were seen (0/1000). Therefore the ability to suppress the p\textsubscript{17} mutation is a property of the original str-1 mutation in 749/110. Other str\textsuperscript{R} mutations have not been tested for their ability to suppress pen p\textsubscript{17}.

749/110\textit{men}03/17 is the only characterized structural gene mutation to undergo a significant change in penicillinase level on transfer from a str-1 to a str-1\textsuperscript{+} environment, though the phenotype of at least one other penicillinase mutant is affected by the transfer from a str-1 to a str-1\textsuperscript{+} environment (Section 3c (ii)).

It has subsequently been shown that both 749/110\textit{men}03/17 (str-1) and 9945A \textit{sad} \textit{pen} \textit{icp}_{17} str-1\textsuperscript{+} can be phenotypically suppressed by streptomycin, kanamycin and 5-fluorouracil, the penicillinase levels being increased up to 10-fold in each case (Collins, personal communication).
3. CHARACTERIZATION OF OTHER PENICILLINASE MUTATIONS

a) Introduction

A number of other penicillinase mutants, derived from strain 749 were studied. All of these mutants were isolated after mutagenesis of 749/110 and were impaired in their ability to be fully induced.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Penicillinase level (U./mg. dry wt. bacteria)</th>
<th>CRM reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>749/110\text{pen}23</td>
<td>24 Uninduced, 23 Induced</td>
<td>-</td>
</tr>
<tr>
<td>749/110\text{pen}31</td>
<td>9 Uninduced, 10 Induced</td>
<td>-</td>
</tr>
<tr>
<td>749/110\text{pen}32</td>
<td>208 Uninduced, 208 Induced</td>
<td>+</td>
</tr>
<tr>
<td>749/110\text{pen}27</td>
<td>15 Uninduced, 130 Induced</td>
<td>-</td>
</tr>
<tr>
<td>749/110\text{pen}14</td>
<td>20 Uninduced, 400 Induced</td>
<td>+</td>
</tr>
<tr>
<td>749/110</td>
<td>20 Uninduced, 1200 Induced</td>
<td>++</td>
</tr>
</tbody>
</table>

All the strains except 749/110\text{pen}31 synthesized enzyme indistinguishable from wild-type, judged by substrate profile and the effect of wild-type antiserum on their activity. 749/110\text{pen}31 produces a slightly abnormal penicillinase judged by the effect of antiserum on its activity (Fleming, unpublished).

From a genetic analysis and a study of the reversion properties of these mutants it was hoped to characterize the nature of the mutations responsible for the observed phenotypes.

b) NTG mutagenesis

A study of the penicillinase phenotypes and genotypes arising from mutagenesis of putative regulatory gene mutations, should enable conclusions to be drawn regarding the nature of the original mutations.

In the \text{lac} system mutagenesis of inducible wild-type strains gives
constitutives (i⁻) at a high frequency. Similarly, mutations preventing inducibility (e.g. i⁵, permease⁻) 'revert' to constitutive level at a comparable frequency to the wild-type strain. This behaviour is a general feature of negative control systems. Therefore one might expect uninducible mutations in the penicillinase system to be overridden by a second magno-constitutive mutation, if penicillinase control is of the negative type.

Mutagenesis was as described in Materials and Methods: 6a. All strains tested were grown and mutagenized under the same conditions, with a magno-inducible strain as control.
Table 24. NTG mutagenesis of putative penicillinase regulatory gene mutations. NTG treatment as in Materials and Methods: 6a. All strains were grown and mutagenized under identical conditions, with a magna-inducible strain as control. The results are compiled from either 2 (749/110pen28 and 749/110pen31) or 3 (749/110pen27, 8a str-1 pen+/pen31 and 9945A ade-1 pen+/pen32/L) independent mutagenic treatments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Auxotroph frequency</th>
<th>Frequency of revertants with raised penicillinase levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50-500 U./mg. penicillinase</td>
</tr>
<tr>
<td>749</td>
<td>2-5 x 10^-2</td>
<td>1-20 x 10^-4</td>
</tr>
<tr>
<td>749/110pen28</td>
<td>1-5 x 10^-2</td>
<td>1-5 x 10^-4</td>
</tr>
<tr>
<td>749/110pen27</td>
<td>2-6 x 10^-2</td>
<td>1-20 x 10^-4</td>
</tr>
<tr>
<td>749/110pen31</td>
<td>2-3 x 10^-2</td>
<td>1-10 x 10^-4</td>
</tr>
<tr>
<td>*8a str-1 pen+/pen31</td>
<td>1-3 x 10^-2</td>
<td>0.5-5 x 10^-4</td>
</tr>
<tr>
<td>**9945A ade-1 pen+/pen32/L</td>
<td>2-4 x 10^-2</td>
<td>&lt;10^-5 (0 found)</td>
</tr>
</tbody>
</table>

* str- pen+/ transformant from the cross: 749/110pen31 --> 8a/6. Produces 9 U./mg. penicillinase and inducible (Section 3c (ii)).

** ilv+ pen+/ transformant from the cross: 749/110pen32 --> 8a/6. Produces 20-25 U./mg. penicillinase and is uninducible (Section 3c (iv), Table 31, cross 1).

+/ Penicillinase levels judged by the plate test.
All the mutants tested, except 9945A ade-1 pen+ pen32/L, could be mutated to magno-constitutive phenotype at frequencies comparable to those obtained for the magno-inducible control. No mutations to higher levels of penicillinase were isolated after mutagenesis of 9945A ade-1 pen+ pen32/L. A number of revertants were assayed in liquid culture. The results of the assays are shown below.

Table 25.

<table>
<thead>
<tr>
<th>Strain</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninduced</td>
<td>Induced</td>
</tr>
<tr>
<td>749/110pen28</td>
<td>3250</td>
<td>3570</td>
</tr>
<tr>
<td></td>
<td>3700</td>
<td>3640</td>
</tr>
<tr>
<td>749/110pen27</td>
<td>4360</td>
<td>4650</td>
</tr>
<tr>
<td></td>
<td>2190</td>
<td>5540</td>
</tr>
<tr>
<td>749/110pen31</td>
<td>1163</td>
<td>1871</td>
</tr>
<tr>
<td></td>
<td>3450</td>
<td>3680</td>
</tr>
<tr>
<td>8a str-1 pen+ pen31</td>
<td>4325</td>
<td>4230</td>
</tr>
<tr>
<td></td>
<td>2420</td>
<td>4780</td>
</tr>
</tbody>
</table>

A = revertants of 500-5000 U./mg. penicillinase by plate test (see Table 24)
B = revertants of 50-500 U./mg. penicillinase by plate test (see Table 24)
See Tables 23 and 24 for parental levels.

These results provide strong evidence that regulation in the B.licheniformis penicillinase system is of the negative type; mutations giving a non- or semi-inducible basal level of penicillinase, being overridden by a second mutation, giving an overall magno-constitutive phenotype. A number of the high level revertants isolated after mutagenic treatment were examined genetically (Section 3a).
The failure to revert 9945A pen\textsuperscript{+} pen\textsuperscript{32/L} is discussed later (Section 3e (iv)).

c) Mapping experiments

(1) 749/110pen\textsuperscript{28}

749/110pen\textsuperscript{28} produces 24 U./mg. of apparently normal penicillinase and is uninducible (Dubsau and Pollock, 1965). High level penicillinase producers were isolated at high frequency after NTG mutagenesis (Section 3b).

A number of mapping experiments were undertaken in an attempt to locate the mutation (or mutations) responsible for the observed phenotype. The results from these experiments are shown in Table 26.

The failure to generate penicillinase recombinants of high level (>25 U./mg.) in crosses 1, 2 and 5, suggests that the strain does not carry an \( j \) mutation (conferring magno-constitutivity). The recombination data from cross 2, indicate that \( r_{28} \) lies distant to \( p_{26} \) (18\% recombination). The failure to generate pen\textsuperscript{i+p\textsuperscript{+}} recombinants in crosses 3 and 4, suggests that \( r_{28} \) is close to the pen \( i_0 \) and \( i_{03} \) mutations.

Results from the NTG reversion experiment suggest that this mutant mutants to magno-constitutivity at a comparable frequency to a pen \( i^+ p^+ \) strain. One of the magno-constitutive revertants obtained (RV1, producing 3250 U./mg. penicillinase, Table 25) was used as a donor in transformation to see if the \( r_{28} \) mutation and the presumed \( i \) mutation introduced by NTG mutagenesis, could be separated by recombination.

The data from Table 27 show that the NTG revertant (749/110pen\textsuperscript{28}/RV1) carries two penicillinase mutations resolvable by recombination. From these results it is impossible to deduce the position of pen \( r_{28} \) with respect to the structural gene and the new \( i \) mutation (\( i_{RV1} \)). If \( r_{28} \) lies between \( i_{RV1} \) and \( p \), it is close to \( i_{RV1} \) (1/67 x 100 = 1.5\% recombination), however if \( r_{28} \) is on the \( p \) distal side of \( i_{RV1} \), it could
**Table 26.** Mapping experiments to characterize 749/110pen26.

**Donor: 749/110pen28**

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Cross</th>
<th>Selected marker (X)</th>
<th>Transforms/ml.</th>
<th>pen X^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 9b/2</td>
<td>i^p_{72} \times r_{26}^P</td>
<td>ile</td>
<td>11700</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 8a/i^p_{26}</td>
<td>i^p_{26} \times r_{26}^P</td>
<td>ilv</td>
<td>4300</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 8a/6</td>
<td>i_{G^P_{22}} \times r_{26}^P</td>
<td>ilv</td>
<td>1147</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. 8T/7</td>
<td>i_{G^P_{72}} \times r_{26}^P</td>
<td>ilv</td>
<td>1890</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. 8a/1</td>
<td>i^p^+ \times r_{26}^P</td>
<td>ilv</td>
<td>3120</td>
<td>about 80</td>
</tr>
</tbody>
</table>

About 20 U./mg., inducibility not tested.

About 20 U./mg., of these 12/65 inducible, 53/65 uninducible.

About 20 U./mg., of these 10/10 uninducible.

About 20 U./mg., of these 10/10 uninducible.

All about 20 U./mg., inducibility not tested.

749/110pen28 was assigned the penicillinase genotype $r_{26}^P$ on the basis that the penicillinase produced is normal (i.e. $p^+$). The designation $p$ indicates an undefined regulatory lesion.
be more distant, a quadruple crossover being required to generate the $r_{28p}^+$ recombinant.

<table>
<thead>
<tr>
<th>Recipient: 8c/1$^+p_{26}$</th>
<th>Transformants/0.8ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor: 749/110pen28/RV1</td>
<td>$\text{ilv}^+$</td>
</tr>
<tr>
<td>(3250 U./mg. penicillinase).</td>
<td>$\text{ilv}^+\text{pen}^+$</td>
</tr>
<tr>
<td></td>
<td>2780</td>
</tr>
<tr>
<td></td>
<td>60 $i_p^+$</td>
</tr>
<tr>
<td></td>
<td>(about 3000 U./mg.)</td>
</tr>
<tr>
<td></td>
<td>6 $i_p^+$</td>
</tr>
<tr>
<td></td>
<td>1 $r_{28p}^+$</td>
</tr>
</tbody>
</table>

All magno-constitutive penicillinase transformants produced the same penicillinase level, judged by the plate test.

From the data in Table 27, it can also be calculated that either $i_{RV1}$ or $r_{28}$ (whichever is nearer to $p_{26}$) is $6/67 \times 100 = 9\%$ recombnable with $p_{26}$. This result should be compared to 18% recombination obtained between $p_{26}$ and $r_{28}$ (Table 26, cross 2). It therefore seems most probable that the marker order is $r_{28} \longrightarrow i_{RV1} \longrightarrow p_{26}$. However more recombinants need to be analysed from more crosses before any definite conclusions can be made as regards the map position of $r_{28}$ in relation to other $i$ gene mutations. Nevertheless it seems that $r_{28}$ is located in the same region of the chromosome as $i$ mutations conferring magno-constitutivity. The level of penicillinase produced by strain 749/110pen28 was altered neither by the presence of $\text{str}-1$ nor by phenotypic suppression.

(ii) 749/110pen31

Strain 749/110pen31 produces 9 U./mg. penicillinase and is uninducible. 749/110pen31 is $\text{CRM}^-$ and retains 95% of its original
penicillinase specific activity in the presence of a standard quantity of wild-type antiserum (wild-type penicillinase retains 65% of its activity under these conditions) (Fleming, unpublished). These observations indicate that the strain is producing low amounts of slightly abnormal enzyme. 749/110pen31 was assigned the pen genotype: pen \( x_{31} p_{31} \) (\( r_{31} \) = uninducibility locus, \( p_{31} \) = putative structural gene mutation). 749/110pen31 was used as the donor in a number of transformation experiments (Table 28).

No transformants having a donor penicillinase phenotype (9 U./mg. and uninducible) have been isolated (0/205 tested). Most pen transformants had a wild-type pen \( i^{+} p^{+} \) phenotype when a \( \text{str}^{-} i^{+} \) recipient was used (Table 28, crosses 1, 2 and 3). The frequencies of magnococonstitutive recombinants were consistent with a donor of wild-type penicillinase genotype. Transformants producing 9 U./mg. of penicillinase were uncommon using \( \text{str}^{-} i^{+} \) recipients; on testing all such transformants were found to be \( \text{str}^{R} \) (749/110pen31 is \( \text{str}^{R} \)) and inducible. Using a \( \text{str}^{R} \) recipient (8a/7 \( \text{str}^{-} i^{+} \), cross 4), the majority of pen transformants produced 9 U./mg. of penicillinase and were inducible. Other pen transformants were \( i^{-} p^{+} \) recombinants.

These results confirm the fact that 749/110pen31 carries at least two penicillinase mutations. One (or more) of these \( r_{31} \) is unlinked to the penicillinase region and is responsible for the lack of inducibility in the parent strain.

The failure to transform this uninducibility character from 749/110pen31 to a 9945A recipient may be because it results from a number of mutations unlinked to each other and unlinked to the penicillinase genes. Another possibility is that the uninducibility can only be expressed in the 749 background. Even so one might expect to be able to transform the relevant genetic information to give the
Table 26. Mapping experiments to characterize 749/110\_pen\_31.

**Donor:** 749/110\_pen\_31 (produces 9 U./mg. penicillinase and uninducible)

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Cross</th>
<th>Transformants/ml.</th>
<th>ilv^+</th>
<th>ilv^+_pen^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 8a/6</td>
<td>(i_{03}^p22 \times r_{31}^p31)</td>
<td>1980</td>
<td>9 (i_p^-)</td>
<td>3000-4800 U./mg. penicillinase (3 tested) about 20 U./mg. and inducible about 9 U./mg. and inducible. (\text{Str}^R)</td>
</tr>
<tr>
<td>2. 8a/7</td>
<td>(i_p72 \times r_{31}^p31)</td>
<td>1760</td>
<td>7 (i_p^-)</td>
<td>about 20 U./mg. and inducible</td>
</tr>
<tr>
<td>3. 8a/1^+_p_26</td>
<td>(i^+p_{26} \times r_{31}^p31)</td>
<td>3210</td>
<td>740 (i_p^+)</td>
<td>about 20 U./mg. and 74/74 inducible about 9 U./mg. and inducible. (\text{Str}^R)</td>
</tr>
<tr>
<td>4. 8a/7 (\text{str}^-1)</td>
<td>(i_0p_{72} \times r_{31}^p31)</td>
<td>956</td>
<td>25 (i_p^+)</td>
<td>about 9 U./mg. and inducible</td>
</tr>
</tbody>
</table>

* Phenotypically wild-type, however if \(\text{str}^-1\) is introduced by transformation, the penicillinase specific activity is reduced to 9 U./mg. (2/2 tested).

The difference in penicillinase level, \(\text{str}^-1\ \ i^-p^+\) recombinants (crosses 1 and 2) and \(\text{str}^R\ \ i^-p^+\) recombinants (cross 4), could not be distinguished by the plate test.
uninducible phenotype after long exposures to highly saturating DNA concentrations. In a transformation experiment in which the recipient, 8a/7 strep-1, was transformed with 10 µg./ml. of 749/110 pen31 DNA for 3 hours, 86/86 penicillinase transformants producing 9 U./mg. penicillinase, were inducible.

The mutation in the penicillinase region (p31) has not been accurately located. In the presence of strep-1, this mutation gives a penicillinase level of 9 U./mg., while in a wild-type strep-1+ background the penicillinase level is indistinguishable from wild-type level (20 U./mg.). Str-1 magno-constitutive recombinants containing p31, produce penicillinase of about half the specific activity (U./mg. dry wt. bacteria) of strep-1+ pen-1 p31 transformants (Table 28). When strep-1+ pen-1 p31 strains (producing about 20 U./mg. penicillinase) are made strep-1 by transformation the penicillinase level is reduced to about 9 U./mg. Wild-type (pen-1 pen+1) genes are unaffected by the presence of strep-1.

The difficulty in distinguishing strains containing p31 from wild-type has made the location of the pen p31 mutation difficult; though the type of cross in Table 28, cross 4, where a strep-1 recipient is used, should allow the mutation to be located. No recombinants were observed between pen p72 and p31 (0/28). The fact that penicillinase produced by strain 749/110 pen31 reacts slightly abnormally with antiserum indicates that the mutation is in the penicillinase structural gene (hence the designation p31). Pen p31 only gives a penicillinase of lowered specific activity in the presence of strep-1.

The penicillinase specific activity of both 749/110 pen31 and 9945A ade-1 pen+ strep-1 pen31 (producing 9 U./mg. penicillinase and inducible, Table 28, cross 4), is increased in the presence of streptomycin, kanamycin or 5-fluorouracil (Collins, personal communication).
The observation that 749/110\text{pen31} can be reverted to a high penicillinase level by NTG suggests that penicillinase regulation is still under the negative control of the i-gene in this strain. One of the high level revertants isolated after mutagenesis of 749/110\text{pen31} was tested in transformation. The revertant chosen (749/110\text{pen31}/RV2) produced 1163 U./mg. penicillinase uninduced and 1871 U./mg. induced, (Table 25). DNA was made from the revertant and used to transform 9b/2 (\text{ile1 pen1} i^{+}p_{72}). After transformation ile^{+} transformants were selected and penicillinase transformants scored. Over 90% of the penicillinase transformants produced more than 1000 U./mg. penicillinase judged by the plate test. Seven of these high level transformants were assayed in liquid culture and were shown to produce 1860–2300 U./mg. penicillinase uninduced and 2780–4100 U./mg. after induction. All the transformants assayed were \text{str}^{3}. These results are consistent with 749/110\text{pen31}/RV2 still containing the \text{pen1} p_{31} mutation, which gives a reduced penicillinase specific activity in the presence of \text{str1}. Two low level penicillinase transformants tested had a \text{pen1} i^{+}p^{+} phenotype.

It is interesting to note that 749/110\text{pen31}/RV2 and other high level revertants of 749/110\text{pen31} have significant inducibility (up to 2x), whereas the parent strain is strictly uninducible. This indicates some interaction between the i-gene product and the product (or products) of \text{pen1} p_{31}.

It will be difficult to characterize the \text{pen1} p_{31} mutation (or mutations) further until it can be transferred to a recipient strain into which other penicillinase mutations can be introduced.

(iii) 749/110\text{pen27} and 749/110\text{pen14}

These strains are two of a number of semi-inducible penicillinase mutants isolated (Dubnau and Pollock, 1965). In the absence of inducer both strains produce apparently normal penicillinase at about normal
basal rate. On induction neither of the strains can be fully induced: 749/110pen27 has a fully induced level of 130-150 U./mg., and 749/110pen14 has a maximal induced level of 400 U./mg. of penicillinase. DNA was made from both these strains and used to transform the recipients shown in Table 29. The results suggest that the r mutation is only about 50% linked to the penicillinase structural gene and i-gene. The data from crosses 4 and 6 suggest that r and i are 40 and 47% linked to p72 respectively. If the double mutants r+i and i have a magno-constitutive phenotype (as seems likely, from the NG reversion studies), the results from crosses 1, 2, 3 and 5 suggest that there is 45% linkage between r and i (crosses 1 and 2), 43% linkage between r and i (cross 3) and 41% linkage between r and i (cross 5). From these results it is not possible to determine on which side of i, r and r lie, however it seems probable that they are located together in a different gene to i.

One of the high level penicillinase revertants of 749/110pen27 was analysed in transformation. The revertant chosen (749/110pen27/RV3) produced 4360 U./mg. of penicillinase (Table 25) and was not significantly inducible. DNA was made from this strain and used to transform a 9b/2 recipient. The results shown in Table 30 were obtained.

The data in Table 30, suggest the presence of a second i-gene mutation (i, conferring magno-constitutivity) in strain 749/110pen27/RV3: i and p72 are 12/105 x 100 = 11% recombiable. From this data it is not possible to orientate r with respect to the other mutations.

In the absence of complementation tests or more accurate mapping data it is not possible to conclude definitely that r (and r) lie in a different gene to the i-mutations characterized; however the recombination data strongly suggest that these mutations lie in another
Table 29. Mapping experiments to characterize 749/110pen27 and 749/110pen14.

**Donor: 749/110pen27**

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Cross</th>
<th>Transforms/µl.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 8a/6</td>
<td>* $i_0^3 p_22 \times r_{27}^p$</td>
<td>1945 i&lt;sup&gt;-&lt;/sup&gt; p&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33 $r_{27}^p$&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34 $i^+_p$</td>
</tr>
<tr>
<td>2. 8T/6</td>
<td>$i_0^3 p_22 \times r_{27}^p$</td>
<td>2780 12 i&lt;sup&gt;-&lt;/sup&gt; p&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64 $i^+_p$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>54 $r_{27}^p$&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>3. 8a/7</td>
<td>$i_0^3 p_72 \times r_{27}^p$</td>
<td>304 2 i&lt;sup&gt;-&lt;/sup&gt; p&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 $i^+_p$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 $r_{27}^p$&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>4. 9b/2</td>
<td>$i^+<em>p p_72 \times r</em>{27}^p$</td>
<td>6860 60 $i^+_p$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ile&lt;sup&gt;+&lt;/sup&gt; 40 $r_{27}^p$&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Donor: 749/110pen14**

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Cross</th>
<th>Transforms/µl.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5. 8a/6</td>
<td>$i_0^3 p_22 \times r_{14}^p$</td>
<td>1320 2 i&lt;sup&gt;-&lt;/sup&gt; p&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 $i^+_p$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 $r_{14}^p$&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>6. 9b/2</td>
<td>$i^+<em>p p_72 \times r</em>{14}^p$</td>
<td>5430 62 $i^+_p$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ile&lt;sup&gt;+&lt;/sup&gt; 55 $r_{14}^p$&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Recipient first.
<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Transforms /ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>9b/2</td>
<td>749/110pen27/RV3</td>
<td>ile$^+$</td>
</tr>
<tr>
<td>(ile$^{-}$ pen i$^+_p$)</td>
<td>(4360 U./mg. penicillinase)</td>
<td>ile$^+$pen$^+$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4520</td>
</tr>
<tr>
<td></td>
<td></td>
<td>93 i$^{-}p^+$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2-5 x 10$^5$ U./mg. penicillinase).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>produce about 20 U./mg. penicillinase.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4/12 i$^+_p$ phenotype.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8/12 i$^+_p$ phenotype.</td>
</tr>
</tbody>
</table>

Magneso-constitutive penicillinase transformants all had the same penicillinase level judged by the plate test.
gene, unless the i-gene is very large and i-mutations investigated are restricted to a small region of the gene.

(iv) 749/110pen32

749/110pen32 synthesizes 200 U./mg. of apparently normal penicillinase and is uninducible (Dubnau and Pollock, 1965). DNA made from this strain was used to transform the recipients shown in Table 31.

The results suggest that at least two mutations are responsible for the measured penicillinase level in 749/110pen32. One (or more) of these mutations (r32), is in the penicillinase region and gives the uninducible 20-24 U./mg. level of penicillinase. A mutation (or mutations) unlinked to the penicillinase region increases the penicillinase level to about 200 U./mg. of penicillinase. This unlinked locus (mod-1) (mod = modifying gene) is linked to pen; 11/15% x 100 = 73% linked, (Table 31, crosses 1 and 3), though mod-1 or another locus having the same effect, can be inherited in the absence of pen (8/172 x 100 = 5%, Table 31, crosses 1, 2 and 3).

If pen i-r32 recombinants produce about 24 U./mg. of penicillinase, as seems probable from the failure to mutate strains containing r32 to a higher penicillinase level (Table 24), then the following recombination values can be calculated from the results in Table 31:

\[
\begin{align*}
\text{r32}/p22 &= 4.8/99 = 0.048 \text{ (cross 1)} \\
\text{r32}/p72 &= 4/40 = 0.10 \text{ (cross 2)} \\
\text{r32}/p72 &= 11.3/60 = 0.19 \text{ (cross 3)}
\end{align*}
\]

These results and the fact that there is a slight predominance of i\textsuperscript{+} p\textsuperscript{+} recombinants over i\textsuperscript{-} p\textsuperscript{+}, indicate the marker order below:-
Table 31. Mapping experiments to characterize 749/110pen32.

**Donor:** 749/110pen32 (produces 200 U./mg. penicillinase and uninducible).

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Cross</th>
<th>Transforms/ml.</th>
<th>(\text{ily}^+)</th>
<th>(\text{ily}^+\text{pen}^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 8a/6</td>
<td>(i_{03}p_{22}^+ \times s_{32}p^+)</td>
<td>2670</td>
<td>2 (i^{-p}^+) pen.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>83 pen; about 24 U./mg.; of these 29/30 uninducible, 1/30 inducible.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 pen; about 200 U./mg. and uninducible.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 pen; about 24 U./mg. and uninducible.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 pen; about 200 U./mg. and uninducible 1/7 (\text{sitR}^+).</td>
<td></td>
</tr>
<tr>
<td>2. 9b/2</td>
<td>(i_{72}^+ \times r_{32}p^+)</td>
<td>1230</td>
<td>0 (i^{-p}^+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>35 about 24 U./mg. and uninducible.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 about 200 U./mg. and uninducible.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 (i^+p^+) about 20 U./mg. and inducible.</td>
<td></td>
</tr>
<tr>
<td>3. 8a/7</td>
<td>(i_{072}^+ \times r_{32}p^+)</td>
<td>1920</td>
<td>5 (i^{-p}^+) pen.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>47 pen; about 24 U./mg.; of these 13/15 uninducible, 2/15 inducible.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 pen; about 200 U./mg. and uninducible.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 pen; about 24 U./mg. and uninducible.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 pen; about 200 U./mg. and uninducible.</td>
<td></td>
</tr>
</tbody>
</table>

\(*_{32} = \text{undefined regulatory locus responsible for observed phenotype (equivalent to } r_{32}^\text{mod-1}; \text{ see text).}\)
Neither the *str-1* allele nor phenotypic suppression (Collins, personal communication), had any effect on the phenotypes of strains carrying *pen r₃₂* and *mod-1*.

An experiment was undertaken to determine the effect of *mod-1* on wild-type penicillinae genes. Both an *ilv⁺* transformant (9945A *ade-1 pen mod-1*⁺ *pen r₃₂p⁺*), producing about 24 U./mg. of penicillinase and uninducible (*Table 31*, cross 1), and *8a/1* were used as recipients in transformation. Donor DNA's were:

(a) *8a/1 pen⁺ (mod-1?) str-1* (i.e. a *str R pen⁺ pen i⁺p⁺* transformant from the cross: 749/110*pen32* → *8a/1*).

(b) 9945A *ade-1 pen⁺ str-1 mod-1 pen r₃₂p⁺* (i.e. *ilv⁺ pen⁺ str R* transformant producing 200 U./mg. penicillinase, *Table 31*, cross 1).

These DNA's were used to transform the recipients given above *str R* transformants being selected after transformation. *Str R pen⁺* double transformants were picked from transformant plates and restreaked out, in order to test penicillinase levels after growth. The results of this transformation experiment are shown in *Table 32*.

These results suggest that *mod-1* is specific for *pen r₃₂*, having no effect on wild-type penicillinae genes. Therefore *mod-1* can be considered a suppressor mutation for *r₃₂*, though its specificity may not be confined to *r₃₂*.

An experiment was undertaken to ascertain if the *mod-1* allele is only found in 749/110*pen32*. A 9945A *ade-1 pen mod-1*⁺ *pen r₃₂p⁺* recipient, producing about 24 U./mg. penicillinase and uninducible, was transformed with a number of DNA's (*Table 33*). The results show that other 749 strains and 9945A/WT carry the *mod-1* allele (i.e. the ability to increase the penicillinase level of *mod-1*⁺ *pen r₃₂p⁺* strains from about 24 U./mg. to about 200 U./mg.). The results from *Tables 32* and 33 also show *mod-1* and *pen* to be 90% linked.
### Table 32. The effect of mod-1 on ren i\(^{+}p\)^{+} and ren \(r_{32}p\)^{+}.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>DONOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 8a/1 (mod-1 (llyD1) mod-1 ren (i^{+}p)^{+})</td>
<td>8a/1 (str-1) ren (i^{+}) (mod-1?)</td>
</tr>
<tr>
<td>2. 8a/1</td>
<td>9945A (mod-1) (str-1) ren (i^{+}) mod-1 ren (r_{32}p)^{+}</td>
</tr>
<tr>
<td>3. 9945A (mod-1) ren ren (r_{32}p)^{+} mod-1 (\text{(about } 24 \text{ U.}/\text{mg. penicillinase and uninducible)})</td>
<td>8a/1 (str-1) ren (i^{+}) (mod-1?)</td>
</tr>
<tr>
<td>4. 9945A (mod-1) ren ren (r_{32}p)^{+} mod-1 (\text{and } 20\text{ U.}/\text{mg. penicillinase and uninducible)})</td>
<td>9945A (mod-1) (str-1) ren (i^{+}) mod-1 ren (r_{32}p)^{+}</td>
</tr>
</tbody>
</table>

### Ren \(str-1\) transformants tested.

- 25/25 produced about 20 U./mg. and inducible.
- 18/18 produced about 20 U./mg. and inducible.
- 5/5 produced about 200 U./mg. and uninducible.
- 15/17 produced about 200 U./mg. and uninducible.
Table 73. The effect of a number of \textit{neo}\textsuperscript{+} donor DNA's on the phenotype of a recipient containing \textit{neo} \textit{x}_{32}\textsuperscript{+}.

\begin{tabular}{l|l|l}
\textbf{Recipient:} & \textbf{\textit{neo} \textit{x}_{32}\textsuperscript{+}} & \\
\rowcolor{gray!20} \textbf{Donor} & & \\
\hline
\textit{9945A \textit{ade-1 neo neo \textit{x}_{32}\textsuperscript{+} \textit{ned-1}+}} & & \\
\hline
749/110\textit{neo}3/25 & 17/19 produced about 200 U./mg.: of these 3/3 uninducible. & \\
& 2/19 produced about 24 U./mg. and uninducible. & \\
749\textit{neo}C/72 & 14/15 produced about 200 U./mg.: of these 3/3 uninducible. & \\
& 1/15 produced about 24 U./mg. and uninducible. & \\
9945A/\textit{WT (neo\textsuperscript{+} neo \textit{i}_{32}\textsuperscript{+})} & 14/16 produced about 200 U./mg.: of these 2/2 uninducible. & \\
& 2/16 produced about 24 U./mg. and uninducible. & \\
\end{tabular}

More than 90\% of \textit{ade}\textsuperscript{+} \textit{neo} transormants in these crosses, produced about 24 U./mg. penicillinase and were uninducible.
The penicillinase phenotype in 749/110pen\(^{32}\) is therefore due to the interaction of \textit{mod-1} on \textit{pen} \(r_{32}\). \textit{Pen} \(r_{32}\) gives a basal uninducible level of penicillinase and maps on the i-distal side of \(p\). Strains carrying \(r_{32}\) cannot be mutated to a higher penicillinase level, though \textit{mod-1} (found in \textit{pen}\(^{+}\) strains but not in any \textit{pen} recipient strains examined) can increase the penicillinase level 8-10 times in strains containing \(r_{32}\).

d) Conclusions

An analysis of a number of regulatory gene mutations, giving a basal level of penicillinase expression and impaired in their inducibility, has shown that the regulation of penicillinase synthesis is not solely under the control of the \textit{i}-gene (characterized by mutations conferring magno-constitutivity, \textit{Section 1}). However the effect of all these regulatory mutations, except \textit{pen} \(r_{32}\) can be overridden by a second mutation in the \textit{i}-gene, resulting in a raised penicillinase level.

The \textit{pen} \(r_{26}\) mutation, giving a basal uninducible penicillinase level, probably maps in the \textit{i}-gene and therefore it is assigned the tentative penicillinase genotype \textit{pen} \(i_{26}p^{+}\). The properties of this mutation are analogous to those of \(i^{s}\) mutations in the \textit{E.coli lac}\ system (Wilson \textit{et al.}, 1964), though the properties of \textit{pen} \(i_{26}\) in the hetero-diploid state cannot be studied at present.

\textit{Pen} \(r_{27}\) and \(r_{14}\), both of which give a semi-inducible penicillinase phenotype, are only about 50\% linked to the \textit{pen} \(i\) and \(p\) genes and therefore probably lie in a different gene to \(i\).

749/110pen\(^{31}\) has at least two penicillinase mutations. One of these (\(p_{31}\)), which is probably in the structural gene, has the property of giving apparently wild-type penicillinase in a \textit{str-1}\(^{+}\) strain, but in the presence of \textit{str-1}, the specific activity of penicillinase
produced by a strain carrying $p_{31}$ is almost halved. There is less than 3.6% recombination between pen $p_{72}$ and $p_{31}$. The other mutation (or mutations), pen $r_{31}$, prevents induction. The failure to transfer the uninducibility character from the parent strain to 9945A recipients, indicates that a number of unlinked loci may be responsible for the uninducibility. The observation that pen $r_{31}$ containing strains mutate to a magno-constitutive level of penicillinase with slight but significant inducibility (up to 2-fold), suggests an interaction between the i-gene product and the product (or products) of $r_{31}$.

749/110pen32 carries a mutation pen $r_{32}$, which gives rise to a basal uninducible expression of penicillinase synthesis. The penicillinase level of strains containing $r_{32}$ is increased 8-10 fold in the presence of mod-1, which is 70-90% linked to pen. The mod-1 allele was found in all strains carrying the pen$^+$ allele that were examined. Mod-1 and pen$^+$ could be dissociated in transformation and the effect of mod-1 was specific for strains carrying $r_{32}$; it had no effect on wild-type penicillinase genes. The mapping data indicate that $r_{32}$ lies on the side of the structural gene distal to i. Preliminary protein structural work (Section 4) suggests that the penicillinase operator/promoter region lies on the i distal side of p and it therefore seems possible that $r_{32}$ is a mutation in this region. The fact that a strain carrying $r_{32}$ cannot be reverted to a magno-constitutive penicillinase level is consistent with $r_{32}$ mapping in the operator/promoter region. The nature of the interaction of mod-1 and $r_{32}$ is discussed in the Discussion.
4. PURIFICATION AND PROTEIN CHEMISTRY OF A MUTANT PENICILLINASE

a) Introduction

*B. licheniformis* strain 749/110*penC3*/19 synthesizes a penicillinase of lowered specific activity (90 U./mg. dry wt. bacteria), altered substrate profile, altered response to specific antiserum (Dubnau and Pollock, 1965) and changed pH profile (P. Hill, unpublished). From the genetic analysis the mutation was found to map at one end of the structural gene, distal to the i-gene. By determining the structural alteration in the mutant protein it was hoped to orientate the structural gene with respect to the penicillinase protein and determine the direction of transcription and translation of the structural gene with respect to the i-gene.

b) Purification procedure

The enzyme was prepared by the method developed in this laboratory for the preparation of wild-type exo-enzyme from strain 749*penC* (Meadway, 1969).

40-50% of the penicillinase synthesized by 749/110*penC3*/19 was released as exo-enzyme in liquid culture. A 10 litre batch culture of 749/110*penC3*/19 was grown to a density of 6 mg. dry wt. bacteria/ml. and harvested. The 10 litres of supernatant obtained were calculated to contain about 50 mg. of the mutant penicillinase on the basis of a specific activity of 90 U./mg., compared to a specific activity of 4000 U./mg. dry wt. bacteria for penicillinase produced by 749*penC* and assuming a specific activity of 430 U./μg. pure wild-type penicillinase (Meadway, personal communication).

After dialysis to reduce its ionic strength, the supernatant was passed through a carboxy-methyl-cellulose column at pH 4.8 to absorb the penicillinase. The penicillinase was eluted from the column with ammonium acetate buffer (0.2M., pH 7.0.); more than 95% of the enzyme
was eluted in a total volume of 500 ml. After dialysis, the solution was freeze-dried; the weight of material obtained was 57.3 mg.

This material was subjected to chromatography on a Sephadex G-100 column. The eluate from the column was monitored at 280 nm; more than 90% of the UV absorbing material was found in a single peak at a position characteristic of penicillinase. Fractions containing penicillinase activity were pooled and freeze-dried after dialysis. The weight of material obtained was 44.4 mg. The absorption spectrum of the material was characteristic of penicillinase and a penicillinase assay of the material showed it to have a total activity of $3.2 \times 10^5$ units.

From a measure of the absorbance of the material at 280 nm, it was deduced that the material contained 37.2 mg of penicillinase (a 1 mg/ml solution of wild-type exo-penicillinase has an absorbance of 0.85 at 280 nm and 1 cm. path length). It was assumed that the mutant penicillinase had an identical value.

On this basis the material was $37.2 / 44.4 \times 100 = 84\%$ pure. The specific activity of the mutant penicillinase was therefore:

$$\frac{3.2 \times 10^5}{37.2 \times 10^5} \text{ U./mg. pure protein} = 8.6 \text{ U./mg. pure mutant penicillinase}$$

This figure is $8.6 / 430 \times 100 = 2.1\%$ of the specific activity of wild-type exo-penicillinase and agrees well with the relative specific activities of 749\_penC and 749/110\_penC3/19 in bacterial cultures ($30 / 4000 \times 100 = 2.3\%$).

A sample of the mutant protein was subjected to starch-gel electrophoresis at pH 8.5. Wild-type exo-enzyme was run as a control. After electrophoresis the gel was tested for penicillinase activity and stained for protein. Three bands of penicillinase activity were found in the same position as the three protein bands in the mutant sample. The positions of the bands were identical to those for wild-type enzyme.
and it was concluded that the mutant protein has the same net charge as wild-type enzyme at pH 8.5. No protein contaminants were visible for the mutant protein preparation.

c) Protein chemistry.

2 mg. of mutant protein were acid hydrolysed (6N. HCl, 24 hours at 105°C. in vacuo). The hydrolysate was analysed on an amino acid analyser (Beckman 120C). No significant differences from wild-type were detected from the amino acid composition. It was calculated that the material hydrolysed (2 mg.) contained 1.8 mg. protein.

The remaining material (40 mg.) was performic acid oxidized and digested with chymotrypsin in parallel with an equal quantity of wild-type exo-enzyme. The enzymic digest was separated into peptides on a G-25 Sephadex column. Samples (100 µl.) from fractions containing 280 mp. absorbing material were sequentially spotted transversely across Whatman 3M paper, and the paper electrophoresed at right angles to the applied peptides at pH 6.5. This procedure results in a 2-dimensional peptide map, characteristic for wild-type penicillinase.

Comparison of the peptide maps for wild-type and mutant penicillinase showed an additional high-molecular weight basic peptide present for the mutant protein. This peptide was purified by electrophoresis at pH 3.5 and subjected to N-terminal analysis, followed by quantitative determination of amino acid composition. It was deduced that the structure of the peptide isolated and purified was compatible with the amino acid sequence of wild-type penicillinase from residue 112-124 (Ambler and Meadway, 1969, and Figure 5).

```
112 120 121 124
-Lys-Gln-Ile-Gly-Gly-Pro-Glu-Ser-Leu-Lys-Lys-Glu-Leu-
```

Wild-type penicillinase is normally cleaved between residues 120 and 121 by chymotrypsin, so that two peptides result from this region.
The lack of cleavage between residues 120-121 gives a basic peptide of high molecular weight. The position of the peptide on the map is consistent with this data. Ninhydrin spots, probably corresponding to the two peptides: residues 112-120 and 121-124 were also seen on the mutant peptide map. It therefore seems likely that the chymotrypsin had only partially cleaved the bond between residues 120 and 121, in the mutant protein.

There is therefore no evidence to indicate that the amino acid substitution in the penicillinase synthesized by 749/110penC3/19 is in the peptide isolated and studied above. In the absence of any other difference peptides the project was abandoned. The fact that 749/110penC3/19 penicillinase had no net charge change compared to wild-type protein, lessened the chance of isolating any difference peptides; future studies of penicillinase mutant proteins would best be confined to those with an altered mobility on starch gel electrophoresis.

An analysis of another mutant penicillinase is in progress (Kelly and Sherratt, unpublished). Strain 749penC71 is CRM+ and synthesizes a penicillinase of specific activity 50 U./mg. dry wt. bacteria. After purification the mutant penicillinase was shown to have an altered net charge, judged by its mobility on starch gel electrophoresis at pH 8.5 (1 charge more basic than wild-type penicillinase). Preliminary structural studies have shown an amino acid substitution in the cyanogen bromide peptide: residues 250-264 (Ambler and Headway, 1969, and Figure 5). It therefore seems that there is an amino acid substitution within 7 residues of the C-terminus of the penicillinase. The genetic analysis has shown that pen p71 is close to p72 (less than 2% recombination); p72 is one of the outside markers of the penicillinase structural gene and is proximal to the i-gene. It therefore seems likely
that the end of the structural gene corresponding to the C-terminus of
the protein is adjacent to the i-gene; it therefore follows that the
penicillinase operator must lie distal to the i-gene.

This conclusion must be regarded as tentative as the specific
amino acid substitution in the mutant penicillinase has not yet been
determined, nor has the possibility of other amino acid substitutions
been completely eliminated.
Figure 5. The amino acid sequence of penicillinase from *B. licheniformis* strain 7498enC.

1 Lys-Thr-Glu-Met-Lys-Asp-Asp-Phe-Ala-Lys-
11 Leu-Glu-Glu-Gln-Phe-Asp-Ala-Lys-Leu-Gly-
21 Ile-Phe-Ala-Leu-Asp-Thr-Gly-Thr-Asn-Arg-
31 Thr-Val-Ala-Tyr-Arg-Pro-Asp-Glu-Arg-Phe-
41 Ala-Phe-Ala-Ser-Thr-Ile-Lys-Ala-Leu-Thr-
51 Val-Gly-Val-Leu-Leu-Gln-Gln-Lys-Ser-Ile-
61 Glu-Asp-Leu-Asn-Gln-Arg-Ile-Thr-Tyr-Thr-
71 Arg-Asp-Asp-Leu-Val-Asn-Tyr-Asn-Pro-Ile-
81 Thr-Glu-Lys-His-Val-Asp-Thr-Gly-Met-Thr-
91 Leu-Lys-Glu-Leu-Ala-Asp-Ala-Ser-Leu-Arg-
101 Tyr-Ser-Asn-Ala-Ala-Gln-Asn-Leu-Ile-
111 Leu-Lys-Gln-Ile-Gly-Gly-Pro-Glu-Ser-Leu-
121 Lys-Lys-Glu-Leu-Arg-Lys-Ile-Gly-Asp-Glu-
131 Val-Thr-Asn-Pro-Glu-Arg-Phe-Glu-Pro-Glu-
141 Leu-Asn-Glu-Val-Asn-Pro-Gly-Glu-Thr-Gln-
151 Asp-Thr-Ser-Thr-Ala-Arg-Ala-Leu-Val-Thr-
161 Ser-Leu-Arg-Ala-Phe-Ala-Leu-Glu-Asp-Lys-
171 Leu-Pro-Ser-Glu-Lys-Arg-Ala-Leu-Ile-
181 Asp-Trp-Met-Lys-Arg-Asn-Thr-Thr-Gly-Asp-
191 Ala-Leu-Ile-Arg-Ala-Gly-Val-Pro-Asp-Gly-
201 Trp-Glu-Val-Ala-Asp-Lys-Thr-Gly-Ala-Ala-
211 -----Ser-Tyr-Gly-Thr-Arg-Asn-Asp-Ile-Ala-
221 Ile-Ile-Trp-Pro-----Pro-Lys-Gly-Asp-Pro-
231 Val-Val-Leu-Ala-Val-Leu-Ser-Ser-Arg-Asp-
241 Lys-Lys-Asp-Ala-Lys-Tyr-Asp-Asp-Lys-Leu-
251 Ile-Ala-Glu-Ala-Thr-Lys-Val-Val-Met-Lys-
DISCUSSION
1. Characterisation of the Transformation System

a) Preliminary studies

Using the transformation system in *B. licheniformis* strain 9945A (Thorne and Stull, 1966), it was possible to transform both nutritional markers and the penicillinase genes at frequencies comparable to those reported by Thorne and Stull for the transformation of nutritional markers.

b) A marker linked to the penicillinase genes

To undertake a detailed genetic analysis of penicillinase mutants, it was necessary to have a means of obtaining large numbers of penicillinase transformants. Selection for penicillinase producing cells in *B. licheniformis* is not practicable for a detailed genetic analysis. The relative resistance against penicillin conferred by penicillinase at the single cell level is low, only becoming appreciable at high cell-densities, when penicillinase producing cells release large amounts of enzyme into the culture medium and consequently penicillinase negative cells are protected from the action of penicillin. A two step selection procedure has been used for selecting penicillinase transformants (Dubnau and Pollock, 1965), though the procedure was laborious and could only be used for selecting transformants with a high penicillinase level in the presence of penicillin from a negative recipient.

The best way of obtaining large numbers of penicillinase transformants is to select for a marker linked to the penicillinase genes. This method has been used successfully in the *S. aureus* penicillinase system, where linked plasmid markers (resistance markers to heavy metals and macrolide antibiotics) can be selected after transduction (Novick, 1963). Of the three methods attempted for obtaining a marker linked to *pen* in *B. licheniformis*, one was successful; a random testing of nutritional markers for linkage, resulted in finding *ilyD*1 which was weakly linked to the penicillinase genes.
c) The phased appearance of transformants and variable linkage

Experiments designed to determine the optimal conditions for transformation led to the observation that a phased appearance of ade-1, ilyD1 and pen transformants resulted when transforming DNA was added at different times after dilution of a recipient culture (8a/1) into TM (Sherratt and Collins, 1968). Moreover the linkage of ilyD1 to pen was variable, the linkage being highest when the number of ily+ transformants was a maximum. This behaviour suggests a phasing of the competent cells with respect to the appearance of transformants.

A possible explanation of this behaviour is that the chromosomes of competent cells are terminated uniquely as the cells reach stationary phase in BLSG. On dilution into TM, DNA synthesis is re-initiated synchronously at the chromosome origin. The phased appearance of transformants could then reflect the integration of DNA at a specific position on the replicating genome at any time. Bodmer (1965 and 1966) has suggested that B.subtilis transforming DNA is integrated at replicating points. If this were true for B.licheniformis, the phased appearance of transformants might reflect the synchronous replication of the chromosome from ade-1 (near the replication origin of B.licheniformis; Tyeryar et al., 1968) to ilyD1/pen (terminal markers; Tyeryar et al., 1968, and results this thesis).

Numbers of transformants are maximal for a given marker when DNA carrying this particular marker can be integrated immediately. At other times DNA must remain associated with the cell in a state insensitive to external DNA'se until it can be integrated. During this time the DNA is subjected to degradation, possibly by nucleases, resulting in lowered numbers of transformants and lowered linkage between linked markers.

However such a hypothesis is difficult to reconcile with all the
experimental facts. If the major peak of ilyD1 transformants appears after about 200 min. in TM, this implies that a replicating point has traversed the whole genome in this time. However, during this time, there has been less than a 20% net increase in DNA in the recipient culture. This net increase in DNA need not reflect DNA synthesis in the competent population; competent cells may be preferentially synthesizing DNA, or DNA precursors may be regenerated from the nucleic acids of dying cells. Nor does this hypothesis explain the early peak of ilyD1 transformants, unless the peak results from the integration of DNA at replicating points of chromosomes that are still being completed.

There is no evidence from the experiments reported in this thesis that the chromosomes of B.licheniformis (8a/1) are completed on reaching stationary phase in BLSG. However, the results of Tyeryar et al., (1968), using derivatives of B.licheniformis strain 9945A, suggest that spores have completed chromosomes and stationary phase cells have chromosomes approaching completion. B.subtilis strain W23 has chromosomes which are completed on reaching stationary phase, whilst strain 168 has chromosomes which are only terminated synchronously on amino acid starvation (Archer and Landman, 1969a). It is not known what limits growth of B.licheniformis (8a/1) cultures in BLSG, though by analogy with other systems competent cultures would not be expected to be amino acid limited, as such a state does not allow the onset of competence in other bacteria (Archer and Landman, 1969a).

Clearly, to study the phased appearance of transformants in B.licheniformis, defined conditions for obtaining competent cells are required as well as a means of studying events in competent cells, rather than the recipient population as a whole. In B.subtilis, defined conditions for obtaining competence have recently been described (Bott and Wilson, 1968), and means of enriching for competent
cells have been reported (Hadden and Nester, 1968; Archer and Landman, 1969a). The fate of labelled transforming DNA after uptake by competent cells can also be studied (Fox and Allen, 1964; Venema et al., 1965; Bodmer, 1965 and 1966), though the results of such experiments are often difficult to interpret. The application of such techniques to *E. licheniformis* should enable a detailed investigation of factors affecting transformation, especially variable linkage and the phased appearance of transformants.

Recently a phased appearance of transformants has been reported for *B. subtilis* (Erickson and Braun, 1968; Cooper and Evans, 1968). Erickson and Braun used a method similar to that used for *E. licheniformis* for obtaining competence. Recipient cultures were grown from spores to stationary phase in a minimal medium supplemented with yeast extract (0.1%). After reaching stationary phase, the cells were diluted into fresh medium of the same composition, where competence developed. A phased appearance of transformants for *ade, thr, met, leu* and *arg* markers was observed and the order of appearance of the various classes of transformants corresponded to their positions on the *B. subtilis* genetic map. DNA prepared from recipient cultures at various times in the dilution medium showed a sequential enrichment for replicated markers, based on a marker frequency analysis (Sueoka and Yoshikowa, 1963). From these results it was concluded that DNA uptake and integration are dependent on DNA replication, integration occurring in a region associated with the replicating point. These workers assumed the replicating point to be associated with the cell-membrane, as has been suggested by a number of workers (Geneser and Lederberg, 1965; Lark, 1966; Jacob et al., 1963).

It was also shown that the frequency of appearance of unlinked double transformants was consistent with a sequential integration of
markers. Variable linkage between linked markers was not reported. Details of the experiments of Cooper and Evans are not available.

These conclusions are in conflict with those of Archer and Landman (1969,a and b), who investigated the appearance of transformants in a thy⁻ trp⁻ strain of B.subtilis in which chromosome replication had been arrested at the terminus by tryptophan starvation. If these cells were now thymine starved and tryptophan added back, the cells could be transformed, though DNA synthesis could not be re-initiated. Such cells were transformed with a thy⁺ trp⁺ fu-r (fluorouracil resistant) DNA for 25 min. and then made into protoplasts and treated with DNA'ase to remove DNA external to the cell membrane. The relative proportions of the different classes of transformants (derived from the protoplasts) were not significantly different to those obtained using an unsynchronized control recipient culture. From this data it was concluded that DNA uptake is not associated with DNA replication at the cell membrane. Archer and Landman also considered their results to be inconsistent with integration at a replicating point, unless non-integrated DNA can remain preserved in the cell until it can be integrated. Some recent experiments with B.subtilis suggest that this may be so; transformation can be inhibited by the addition of specific anti-DNA antibodies after the transforming DNA has become insensitive to external DNA'ase (Erickson et al., 1968).

It therefore seems that many of the features concerning the phased appearance of transformants in B.licheniformis are common to a similar phenomenon in B.subtilis. In both cases the appearance of transformants seems to reflect the replication order of the genes on the respective genetic maps. At present, there is no direct evidence to suggest that transforming DNA is integrated at replicating points; however, techniques are becoming available that should allow conclusive studies
to be made regarding the nature of DNA integration in transformation and other methods of genetic transfer.

d) Fine structure mapping

The results of 2- and 3-factor crosses within the penicillinase region have allowed most penicillinase mutations to be placed in an unambiguous linear order (e.g. see Figure 4). Recombination values were reasonably additive and the recombination values calculated from a cross and its reciprocal were usually comparable. 3-Factor crosses gave an unambiguous order for those markers tested, though the frequency of quadruple crossovers was higher than theory predicted. Future mapping experiments would be made easier if closely linked markers were available on each side of the penicillinase genes for the selection of transformants.

Transformational analysis in B. licheniformis can only be used for ordering markers relatively close together; mutations in the penicillinase genes could be ordered by transformation, but the orientation of pen i and p genes with respect to ilyD1 and ile-1 was not possible. The use of a transduction system giving high linkage values (e.g. using phage SP-15; Tyerar et al., 1968) should enable the ordering of markers unlinked by transformation. Such a transduction system would not be very useful for a fine structure analysis of pen because of the low recombination frequencies expected.

There is 13% recombination, using transformation, between the two most distant markers (p22 and p72) in the penicillinase structural gene; if p22 and p72 give amino acid changes near the ends of the penicillinases synthesized, then the 13% recombination represents about 780 base pairs (i.e. 0.05% recombination per triplet and 0.017% recombination per base pair). In contrast, there is 0.2% recombination between an i and p mutation on a staphylococcal penicillinase plasmid.
In a transformational analysis of $trpB$ mutants in *B. subtilis* (Carlton, 1966), the most distant sites were 20% recombinable and if the molecular weight of *B. subtilis trpB* protein is about the same as *E. coli* trpB protein (50,000; Goldberg *et al.*, 1966), then the recombination values obtained by transformation in *B. subtilis* and *B. licheniformis* are clearly comparable.

The results of a fine structure transformational analysis of the $trpB$ region of *B. subtilis* (Carlton, 1966) gave an unambiguous marker order and reasonably additive map distances using 2- and 3-factor reciprocal crosses. Fine structure analysis of the *amiA* region in pneumococcus is complicated because recombination values are dependent on the nature of the mutant sites in the donor and recipient; recombination values obtained from reciprocal crosses are often widely different (Sicard and Ephrussi-Taylor, 1965).

It is concluded that transformational analysis in *B. licheniformis* is a reliable method of genetic mapping for relatively small genetic distances (up to three average sized genes); for larger distances a transduction system giving higher linkage values than transformation promises to be the most useful tool.

### 2. The Location of the Penicillinase Genes on the *B. licheniformis* Genetic Map

It is clearly desirable to locate the penicillinase genes on the *B. licheniformis* genetic map and to find other markers closely linked to the *pen* genes. This would facilitate genetic mapping and increase knowledge of the *pen* region of the genetic map.

The finding that ile-1, met-1 and the 'aromatic' cluster of genes are all linked to *ilyD1*, confirms that the *B. licheniformis* genetic map is very similar to that of *B. subtilis* (Tyeryar *et al.*, 1968 and
Appendix III) and that these markers map in the terminal region of the chromosome. Possible differences between *B. licheniformis* and *B. subtilis*, in these terminal chromosomal regions, are that no thy mutations have been found between *ilyD* and *ile-1* in *B. licheniformis* though the linkage values indicate that there is room for at least one other average sized gene between *ilyD* and *ile-1*. Preliminary mapping results also suggest that the aromatic gene clusters in the two organisms may be orientated in opposite directions with respect to *ilyD* and the terminus. The marker frequency analysis by Tyeryar et al. (1968) of the *B. licheniformis* chromosome also indicates that *tyr* is replicated before *try* (in agreement with the results in this thesis); however, the resolution of marker frequency analysis is insufficient to give a definitive order for linked markers. No penicillinase has been characterized in *B. subtilis*, though some strains have been reported to produce a penicillinase-like activity (Hill, personal communication). *Mat-1*, the terminal marker studied by Tyeryar et al. in their marker frequency analysis, is 15% linked to *ile-1*. Tyeryar et al. showed *mat-1* to be 2% linked by transformation to a *try* mutation; however using transduction with phage SP-15, these markers were 63% linked. Therefore *ilyD* and *pen* would be expected to be closely linked using SP-15 transduction. The use of this transduction system promises to be useful for extending the genetic map: linkage should be detected between markers showing no linkage by transformation. From the results presented in this thesis, and those of Tyeryar et al. (1968), it is concluded that *pen* is a terminal marker in *B. licheniformis*, being replicated after *mat-1*, *ile-1* and *ilyD* (Appendix III). No markers replicated after *ile-1* have been reported for *B. subtilis*.

In view of the location of *pen* on the *B. licheniformis* genetic map and by comparison with the *B. subtilis* genetic map, it is interesting
to note the location of an E. coli chromosomal penicillinase gene, \textit{empA} (Appendix III; Taylor and Trotter, 1967). \textit{AmpA} maps at 80.5 min. (Taylor and Trotter, 1967) and nearby are mutations conferring resistance to D-cycloserine (77.7 min.). To one side of \textit{empA} are \textit{ilv/met/ile} loci (74-77 min.) while on the other side of \textit{empA} is \textit{murA} (81 min.). If the \textit{B.licheniformis} map is circularized, the \textit{ilv/ile/met} loci lie to one side of \textit{pen}, while \textit{ade-1} (i.e. a \textit{mur} locus) is on the other side of \textit{pen} (see Appendix III). It is difficult to decide if this is a significant similarity between the \textit{B.licheniformis} and \textit{E. coli} chromosomes.

To generate more mutations linked to \textit{pen}, the \textit{in vitro} mutagenesis of transforming DNA (or transducing phage), followed by testing of either \textit{pen} or \textit{ilv} transformants for induced mutations, promises to be the most successful way. The only linked mutation in \textit{B.licheniformis}, obtained by this procedure, is \textit{trnE1} (linked to \textit{ilvD1}), though judging by the success of this procedure for obtaining linked mutants in \textit{B. subtilis} (e.g. see Barat et al., 1965), it ought to be possible to generate more linked mutations in \textit{B.licheniformis} by this means.

3. Suppression

The fortuitous discovery in \textit{B.licheniformis} of a suppressor mutation (\textit{str-1}) should allow a more complete characterization of mutations and a more detailed analysis of gene function.

The best characterized mutation suppressible by \textit{str-1} is \textit{pen} $p_{17}$, which is probably a nonsense mutation in the penicillinase structural gene (Discussion; 4b). The extent of suppression of $p_{17}$ by \textit{str-1} is small (about 28/4000 x 100 = 0.7% of wild-type); the extent of phenotypic suppression of $p_{17}$ (in either a \textit{str-1} or \textit{str-1}$^+$ background), is about the same. Natural genetic suppression of $p_{17}$ in a \textit{str-1}$^+$
background is about 10% of that in the presence of \textit{str-1}. The efficiency of suppression of \textit{p17} by \textit{str-1} is lower than that of tRNA suppressors in \textit{E.coli}: ochre suppressors give up to 5% suppression, while amber and UGA suppressors can give more than 50% suppression (Kaplan \textit{et al.}, 1965; Brenner and Beckwith, 1965; Sambrook \textit{et al.}, 1967).

The finding that the suppressor locus is apparently identical to \textit{str-1}, a mutation conferring resistance to more than 100 mg/ml streptomycin, indicates that the suppression is the result of translational misreading on the ribosome (mutations to high streptomycin resistance have altered 30s ribosomal protein, Traub and Nomura, 1968). \textit{Str-1} is therefore analogous in some ways to the \textit{ram} (ribosomal ambiguity) mutations in \textit{E.coli} (Rosset and Gorini, 1969). \textit{Ram} mutations give rise to altered 30s ribosomal subunits and suppression by \textit{ram} is increased in the presence of streptomycin.

\textit{Str-1} and \textit{ram} differ in that \textit{str-1} confers high resistance to streptomycin, while \textit{ram} mutants are hypersensitive to streptomycin and moreover, suppression by \textit{ram} is antagonized in the presence of streptomycin resistance mutations at the \textit{strA} locus.

The complete specificity of suppression by \textit{ram} has not been determined. All three nonsense mutations can be suppressed, suggesting lack of base specificity in suppression. Suppression of missense mutations has not been reported, though \textit{ram} containing strains grow more slowly than wild-type strains unless the effect of \textit{ram} is restricted by the presence of \textit{strA} mutations.

The specificity of suppression by \textit{str-1} is unknown. \textit{Str-1} has no observable effect on a number of penicillinase structural gene mutations. Penicillinase synthesized by strains carrying a wild-type structural gene and \textit{str-1}, is identical enzymologically to that synthesized by wild-type \textit{str-1}\textsuperscript{+} strains. The penicillinase produced by
strains containing ren p_{17} and str-1 also appears to be identical to wild-type. These observations suggest any translational misreading caused by str-1 is not sufficient to alter the enzymological and serological properties of penicillinase. There is therefore some specificity of suppression by str-1. It would be of interest to isolate penicillinase revertants from strains carrying unsuppressed (str-1^+) ren p_{17}; a large number of these would be expected to be suppressor mutations if p_{17} is a nonsense mutation. External suppressors should include tRNA suppressors of high specificity and which could be used to characterize nonsense mutations and to test the specificity of str-1. It would also be interesting to test other str^R mutations for suppressing activity.

Because of the ability of str-1 to suppress, it is interesting to note that B.licheniformis ribosomes had the highest sedimentation coefficient (S) amongst 25 species of different bacterial ribosomes examined (Taylor and Storck, 1964), though the S values for the ribosomal RNA's of B.licheniformis showed no significant differences to those obtained for other bacterial species (Taylor et al., 1967). In contrast, the ability to transform str^R mutations between B.subtilis and B.licheniformis (Dubnau et al., 1965; Goldberg et al., 1966) indicates that str genes in the two organisms have reasonable homology. In view of the suppressing ability of str-1 it would be interesting to introduce this mutation into B.subtilis and observe its effects on suppressible mutations in this organism.

There have been other reports of mutations simultaneously giving rise to an alteration in ribosome structure and giving suppressing activity (Apixion, 1966; Gartner et al., 1969). In one of these cases, it has been shown that both suppression and ribosomal alteration result from an ochre suppressor mutation giving an altered tRNA. It
therefore seems that the altered ribosomes are a secondary product of a mutation resulting in a changed tRNA; the nature of the change in the tRNA species is not known (Gartner et al., 1969). In the other case (Apirion, 1966), the primary mutation was shown to be in the 50s ribosomal component and the mutation suppressed ( trxA3) was a missense mutation.

4. The Penicillinase Structural Gene

a) Structural gene mutations giving a CRH+ protein, having altered enzymological and immunological properties

Most strains carrying penicillinase structural gene mutations are CRH+ and produce a penicillinase of altered enzymological properties. Only two structural gene mutations (p22 and p72) having no detectable penicillinase activity have been characterized. These two mutations are at opposite ends of the structural gene and at present define its boundaries. All other mutations mapping within p22 and p72 produce detectable penicillinase activity and they map uniformly throughout the structural gene. A number of such mutations have not been recombined with p22 and p72; giving a penicillinase of about 2% the specific activity of wild-type, has not been recombined with p22 (less than 0.5% recombination) and p71 and p20 have not been recombined with p72 (less than 2% recombination). It therefore seems possible that p22 and p72 are small deletions or multi-site mutations. NTC, the mutagen used to generate the p22 and p72; is known to give a high frequency of deletions and multi-site mutations. CRH+ structural gene mutants producing detectable penicillinase activity are probably missense mutations, arising from base substitutions; the majority of such mutants were unaffected either by the presence of str-1 or by phenotypic suppression (Collins, personal communication).
However two putative missense mutations were subject to suppression. 749/1100ppn6/71 has a penicillinase specific activity of 50 U./mg. dry wt. bacteria, is CRI* and produces a penicillinase of altered enzymological properties (Fleming, unpublished). Pen p71 has not been recombined with p72 (less than 2% recombination) and preliminary protein structural studies (Kelly and Sherratt, unpublished), indicate an amino acid substitution near to the C-terminus of the mutant protein. The penicillinase specific activity of 749/pen6/71 is increased in the presence of streptomycin, kanamycin and 5-fluorouracil (Collins, personal communication). However the response is different to that of the apparent nonsense mutations, p17 and p26: the extent of suppression of p71 is far less and only occurs at higher concentrations of suppressing agent when growth inhibition is considerable. The effect of introducing p71 into a str-1 background has not been tested.

749/110ppn31 carries a putative structural gene mutation (p31), which is suppressible by streptomycin in a similar way to p71; suppression only occurs in the presence of high concentrations of suppressing agent, when growth inhibition is appreciable, and the extent of suppression is low. In a str-1 background the penicillinase specific activity of strains containing p31 is reduced by about 50%, and the protein reacts abnormally with antiserum (Fleming, unpublished), indicating an altered penicillinase. In the presence of str-1+, the penicillinase specific activity of strains carrying p31 is indistinguishable from wild-type (though such strains are still subject to suppression, unlike wild-type). The enzymological and immunological properties of the protein synthesized in a p31 str-1+ containing strain have not been studied. Recombination data show that p31 is near to p72 (less than 3.6% recombination); therefore p31 like p71, is probably a missense mutation mapping near the end of p.
corresponding to the C-terminus of the protein. The suppressibility of $P_{31}$ and $P_{71}$ may be a function of the terminal position of these mutations.

Because of the probable positions of $P_{71}$ and $P_{31}$ near to the end of $P$ corresponding to the C-terminus of penicillinase, it is conceivable that the mutations are nonsense mutations, giving a penicillinase a few residues short and having considerable biological activity. However this seems unlikely: protein structural studies of the penicillinase synthesised by a strain carrying $P_{71}$, indicate that the C-terminal residues are present in at least 50% of the amount expected if the whole structural gene is translated (Kelly and Sherratt, unpublished). With $P_{31}$, the fact that the penicillinase specific activity is near normal in a $atr^{-1}$ background and is only reduced in the presence of $atr^{-1}$, again indicates that $P_{31}$ is not a nonsense mutation, unless a penicillinase molecule completely lacking the few terminal residues has normal specific activity whereas a completely translated penicillinase having an amino acid substitution near to the terminus has less activity.

b) Apparent nonsense mutations

Three $G^M^R$ mutants of low penicillinase specific activity (U./mg. dry wt. bacteria) were isolated after mutagenesis of a magno-
constitutive strain (Dubnau and Pollock, 1965); the new mutations, responsible for the observed phenotypes, mapped within the structural gene (Figure 4). Strains carrying two of these mutations ($749/110_{mm3}^3/17$ and $749/110_{mm3}^3/25$) produce normal penicillinase, judged by its reaction with antiserum. It is therefore likely that these strains are producing low amounts of normal penicillinase; mutations with these properties are probably nonsense mutations and the observed penicillinase activity is probably due to a low level of natural suppression (up to
Pen $p_{17}$ is suppressible by $\text{str}^{-1}$ (about 10-fold) and phenotypically suppressible by streptomycin, kanamycin and 5-fluorouracil to a similar extent (Collins, personal communication); these results strongly indicate that the mutation is a nonsense mutation (e.g., see Whitfield et al., 1966).

Pen $p_{25}$ is neither phenotypically (Collins, personal communication) nor genotypically (by $\text{str}^{-1}$) suppressible. The penicillinase level of 749/110penC3/25 (2.8 U./mg.) is similar to that of a $\text{str}^{-1} \cdot \text{pen}^{-1} \cdot \text{i}_{03} \cdot \text{p}_{17}$ strain (2-4 U./mg.) and it seems possible that both $p_{17}$ and $p_{25}$ are nonsense mutations giving 2-4 U./mg. of normal penicillinase in a $\text{pen}^{-1} \cdot \text{i}_{03}$ background, in the absence of suppression. The failure of $p_{25}$ to be suppressed could be the function of the position and nature of the mutation.

749/110penC3/26 has a lower penicillinase specific activity (0.9 U./mg. dry wt. bacteria) than the two strains above and the penicillinase produced has altered enzymological properties. In the presence of streptomycin, kanamycin or 5-fluorouracil, the penicillinase level of 749/110penC3/26 is increased up to 10-fold (Collins, personal communication), though no change in activity on transfer from a $\text{str}^{-1}$ to a $\text{str}^{-1} \cdot \text{i}_{03} \cdot \text{i}_{03} \cdot \text{p}_{17}$ environment has been observed. At these low levels small differences in penicillinase activity might not be distinguished by the iodine/penicillin plate test.

The most consistent explanation of the nature of $\text{pen} \cdot \text{p}_{26}$ is that it is a nonsense mutation, which in the parent strain is suppressed by $\text{str}^{-1}$ to give an altered penicillinase, the amino acid usually inserted at the nonsense triplet giving a penicillinase of reduced specific activity compared to that synthesized by 749/110penC3/17 ($1/20 = 5\%$). In a $\text{str}^{-1} \cdot \text{i}_{03} \cdot \text{i}_{03}$ environment, suppression is lower, but other
amino acids might be introduced at the nonsense triplet to give a lowered synthesis of more active penicillinase and consequently no significant change in specific activity (U./mg. dry wt. bacteria) is observed when a strain carrying \( p_{26} \) is transferred from a \( \text{str}^{-1} \) to a \( \text{str}^{-1}^{+} \) environment.

\( \text{Pen} \ p_{17}, p_{25} \) and \( p_{26} \) all map in the half of the structural gene nearer to \( p_{22}^{+} \) which probably corresponds to the N-terminus of the protein. If these mutations are nonsense mutations, then the fragments of prematurely completed peptides would not be expected to have enzymological or immunological activity. It would be interesting to isolate and characterize revertants from strains carrying these mutations, to see if specific external suppressors can be obtained for these mutations. The characterization of specific nonsense suppressors would enable a classification of nonsense mutations.

5. Regulatory Gene Mutations and the Control of Penicillinase Synthesis in \( \text{B.licheniformis} \)

The regulation of penicillinase synthesis in \( \text{B.licheniformis} \) is under negative control (e.g. see Jacob and Monod, 1961). A gene (i), closely linked to the penicillinase structural gene (p), controls inducibility. In this work, i was first defined by mutations conferring magno-constitutivity (including one mutation temperature sensitive for repression). All these mutations are closely linked, the two distal mutations being 8% recombinable and the nearest i mutation to p is 8.5% recombinable with \( p_{72}^{+} \) the nearest structural gene mutation (\textbf{Figure 4}). No i deletions have been characterized.

The end of p corresponding to the C-terminus of the enzyme, probably lies proximal to i, indicating that the end of p distal to i, is transcribed and translated first. The penicillinase operator (o)
must also be on the i-distal side of p, though it is not known if there are other genes under its control.

The fact that 0 lies on the i-distal side of p could mean that i is part of the penicillinase operon and is under the control of 0. In the absence of complementation studies, this could be investigated by using one of the apparent nonsense mutations in p; these might be expected to be polar and consequently low amounts of i product would be synthesized. This could be tested by constructing an i+ strain carrying a putative nonsense mutation; the penicillinase level produced by such a strain would be higher than expected and should be measurable if the polar effect were strong.

A mutation (i28), giving a basal uninducible level of penicillinase (baso-constitutive) also maps in the i region. Strains carrying i28 can be mutated at high frequency, by a second linked i mutation to give a magno-constitutive phenotype. This observation indicates the negative nature of control in this system. Pan i28 is therefore analogous to the i8 mutations in the E. coli lac system (Wilson et al., 1964), though complementation studies to verify this conclusion are not possible at present in B. licheniformis. The failure to isolate baso-constitutive regulatory gene mutants after mutagenesis of magno-constitutive strains (Dubnau and Pollock, 1965), confirms the negative action of i product (e.g. see Sheppard and Englesberg, 1966). With the discovery of a suppressor system in B. licheniformis, it should be possible to confirm unambiguously the repressive action of i product (A) and its inferred protein nature; though these are strongly indicated by the behaviour of strain 749temT9, which is temperature sensitive for penicillinase repression.

Other mutations, which result in a basal level of penicillinase synthesis and which impair induction in strains carrying these
mutations, map in at least two other regions. Two mutations giving a semi-inducible phenotype \((r_{27} \text{ and } r_{14})\) are only about 50% linked to \(i\) and \(p\). An examination of more mutants impaired in their inducibility, and having a basal level of penicillinase, should show if mutants of semi-inducible phenotype are restricted to the region defined at present by \(r_{27}\) and \(r_{14}\).

*Pen* \(r_{31}\) is a mutation (or mutations) giving a basal uninducible penicillinase phenotype. It has not been possible to transfer this uninducibility character from the strain in which it was generated \((749/110pen^{31})\) to a 9945A recipient. It is therefore probable that either a number of unlinked loci are responsible for the expression of the uninducibility character or that \(r_{31}\) is a single mutation that only can be expressed in a 749 environment. It will be difficult to investigate the properties of \(r_{31}\) further until it can be introduced and expressed in a 9945A recipient.

The phenotypes resulting from \(r_{27}\) and \(r_{31}\) can be overridden by a second \(i\) mutation to give a magno-constitutive phenotype \((r_{14}\) has not been tested for further mutation to magno-constitutive phenotype). Mutations with these properties are most likely impaired in their ability to allow inducer to interact normally with repressor. By analogy with other negative control systems, such mutants might arise by mutation in \(i\), so that inducer no longer interacts with repressor \((i_{28}\) ?), by mutation in a permease which transports inducer into the cell, or by mutation in a component responsible for the conversion of added inducer to true inducer.

From the studies presented here it is impossible to assign definitive roles to the two regions defined by \(r_{27}/r_{14}\) and \(r_{31}\). The observation that some of the high level penicillinase revertants isolated after mutagenesis of 749/110pen^{31} have significant
inducibility (749/110mem31 is strictly uninducible), indicates that there is some interaction between product (R), inducer and the product of the gene containing r\textsubscript{31}. The possible role of these mutations is discussed further when a model of penicillinase regulation is presented (Discussion: 7).

Another mutation (r\textsubscript{32}) gives a basal uninducible level of penicillinase and it has not been possible to mutate a strain carrying r\textsubscript{32} to a higher penicillinase level (less than 1/10\textsuperscript{5} colonies examined) and it is therefore concluded that the regulation of penicillinase synthesis in strains carrying this mutation is no longer under the control of i. Such behaviour might result from a mutation in the penicillinase operator/promoter region. Consistent with r\textsubscript{32} being a mutation in the operator/promoter region is the recombination data, which indicate that r\textsubscript{32} maps on the i-distal side of p (protein structural evidence indicates that the penicillinase operator lies on the i-distal side of p).

The level of penicillinase synthesized by strains containing r\textsubscript{32} is increased about 10-fold in the presence of the mod\textsuperscript{-1} allele, which is 70-90% linked to paa and is present in the parent strain containing r\textsubscript{32} (749/110mem32, producing 200 U./mg. of penicillinase and uninducible). An examination of other strains for the presence of mod\textsuperscript{-1} showed it to be present in the three other paa\textsuperscript{+} strains examined (749/110mem03/25, 749mem0/72 and 9945A/WT). All 9945A paa recipients tested (3/3) are mod\textsuperscript{-1}. The mod\textsuperscript{-1} allele can be dissociated from paa\textsuperscript{+} by transformation, when it still exerts its effect on r\textsubscript{32}. Mod\textsuperscript{-1} has no effect on wild-type penicillinase genes.

Experiments are in progress at present to generate paa\textsuperscript{+} 'revertants' from a 9945A ade\textsuperscript{-1} paa mod\textsuperscript{-1} mem r\textsubscript{32}\textsuperscript{+} strain (24 U./mg. and uninducible). It is hoped to determine what proportion (if any) of
The $\text{pen}^+$ 'revertants' have acquired the $\text{mod}^{-1}$ character.

It is difficult to propose a complete explanation for all these observations. The mapping data and the failure to isolate generate magno-constitutives after mutagenesis of a strain carrying $r_{32}$, are consistent with $r_{32}$ mapping in the penicillinase operator/promoter region. However the phenotype resulting from $r_{32}$ in a $\text{mod}^{-1}$ background (basal penicillinase level, uninducible) is not equivalent to that of either $o^o$ or promoter mutants in the lac system; nevertheless, it is not inconceivable to envisage a mutation in the operator/promoter region giving such properties. The ability of $\text{mod}^{-1}$ to exert a specific control of $p$ in a strain carrying $r_{32}$ might be the result of $\text{mod}^{-1}$ product (a controlling element of another operon ?) interacting with the mutated penicillinase operator/promoter containing $r_{32}$. An alternative possibility is that $r_{32}$ is the result of an event more complex than a single mutation or small deletion. If $r_{32}$ is the result of a transposition or deletion which puts penicillinase regulation under the control of another operon, then $\text{mod}^{-1}$ might be the normal controlling element of this operon. Transformation experiments, using a donor containing $r_{32}$, indicate that the penicillinase genes have not been grossly transposed; the normal frequency of $\text{pen}$ transformants (containing $r_{32}$) was observed when $\text{ilvD1}$ transformants were selected after transformation. It is therefore possible that $r_{32}$ is a deletion which fuses the penicillinase genes onto a neighbouring operon, so that the control of penicillinase synthesis is now under the control of the operator of this operon.

It has been proposed that penicillinase has evolved from a cell-wall synthesizing enzyme and consequently the penicillinase genes might be found linked to markers involved in determining cell-surface structure and properties (Citri and Pollock, 1966). In view of this it
is interesting to note the effect of \( \text{mod-1} \) (closely linked to \( \text{pen} \), a gene determining a cell-surface property) on the penicillinase genes. If \( \text{mod-1} \) is normally involved in determining or regulating some cell-surface property, then its ability to alter the penicillinase level in a strain carrying \( \text{r}_{32} \), indicates that either penicillinase control in such strains is now under the control of an operon specifying some cell-surface property or that mutation in the penicillinase operator/promoter allows interaction with \( \text{mod-1} \) product. In either case, a relationship between the penicillinase genes and other cell-surface markers is established.

Correlation between the penicillinase genes and markers determining cell-surface properties has been demonstrated in other systems. In \( \text{E. coli} \) a chromosomal penicillinase gene (\( \text{emnA} \)) maps in the same region of the chromosome as a number of D-cycloserine resistance markers (Boman et al., 1967; Taylor and Trotter, 1967), while in \( \text{S. aureus} \) a colonial morphology marker maps on penicillinase plasmids (Richmond, 1965).

6. Comparison of Penicillinase Regulation in \( \text{B. licheniformis} \) and \( \text{S. aureus} \)

It is interesting to compare the data regarding penicillinase regulation in \( \text{B. licheniformis} \) and \( \text{S. aureus} \), especially because of the recent finding that the penicillinases synthesized by each of these bacteria are 40% homologous in amino acid sequence (Ambler and Meadway, 1969).

Plasmid-coded penicillinase synthesis, in \( \text{S. aureus} \), is under the negative control of gene \( \text{i} \), which is closely linked to the penicillinase structural gene \( \text{(p)} \); \( \text{i} \) was originally defined by mutations conferring magno-constitutivity. Results from complementation
studies indicated that mutations, which were closely linked to p and conferred defective inducibility on a strain synthesizing the basal level of penicillinase, mapped in a different gene to mutations conferring magno-constitutivity (heterozygous diploids containing magno-constitutive and baso-constitutive or semi-inducible plasmids, gave a fully inducible expression of both structural genes: Richmond, 1967). A genetic analysis of a meso-constitutive mutant (Smith, 1968) also indicated the presence of two genes, one (called $i_N^p$) determining the fully induced level of penicillinase expression, while the other (called $i_R^p$) determined the repressed level. The fact that all putative $i_N^p$ mutations (giving a basal penicillinase level and defective inducibility), when present with $i_R^p$ mutations (conferring magno-constitutivity) in heterozygous diploids, gave a restoration of wild-type inducibility to both structural genes, argues against the possibility of $i_N^p$ and $i_R^p$ mutations mapping in the same gene (complete interallelic complementation is rare, even when a given gene product associates to form a multimer: e.g. see Fincham, 1966). Unfortunately the mutations responsible for defective inducibility and a basal level of penicillinase have not been mapped accurately, though the mutations were shown all to be closely linked to the structural gene. No reversion studies with these mutants have been reported.

It therefore seems that, as in S. licheniformis, mutations giving a basal expression of penicillinase and impaired in their inducibility, may not map in the same gene as mutations conferring magno-constitutivity.

Apart from the plasmid-coded regulatory gene mutations in S. aureus, a chromosomal mutation (R2) which results in a constitutive synthesis of penicillinase in a cell containing the mutation has been described (Cohen and Sweeney, 1968). This chromosomal mutation confers magnoconstitutive synthesis on all plasmid-coded wild-type inducible
genes that are introduced into the cell. However the ability to confer constitutive synthesis was only evident at 32°C; at 40°C, the level of penicillinase synthesis in the presence of R2 mutations was much lower. Plasmid constitutive mutations (1^c) gave rise to a constitutive level of penicillinase synthesis at both 32°C and 40°C. This temperature dependent effect of R2 and the observation that strains containing R2 produce small colonies, indicate that the effects of R2 may not be limited to the constitutive synthesis of penicillinase; it is possible that the mutation affects some property of the plasmid (or cell) as a whole, one of the effects leading to the constitutive synthesis of penicillinase. No mutations unlinked to the penicillinase genes in B. licheniformis give a raised penicillinase level.

Other apparent regulatory gene mutants which synthesise low levels (less than basal) of apparently normal penicillinase, have been isolated (Richmond, 1966 and Smith, 1968). Some of the mutants were inducible (micro-inducible) while others were constitutive (micro-constitutive). All the mutations responsible for these phenotypes mapped on the plasmid and were expressed in the cis-position in heterozygous diploids. A gene product of the penicillinase genes carrying the 'micro-mutation' was able to restore inducibility to a plasmid synthesizing a magno-constitutive level of penicillinase in the trans-position.

In the absence of mapping data, there is insufficient evidence to completely characterize the nature of the mutations involved. Richmond considered that the micro-inducible mutations might be either promoter mutations or structural gene mutations, though he excluded the micro-constitutive mutations from mapping in the structural gene on the basis of the simultaneous drop in activity and loss of inducibility. A
detailed analysis of a micro-constitutive mutation by Smith (1968) showed that the micro-constitutive phenotype was due to two mutations, one in \( i_N \) giving a basal uninducible level of normal penicillinase and a second mutation in the structural gene giving a reduction in specific activity of the penicillinase synthesized.

Judging by the frequency of apparent nonsense mutations in the \( B. licheniformis \) structural gene (3/11 examined), one might expect a number of the 'micro-mutants' investigated by Richmond (1966) to be nonsense mutations in the structural gene.

Penicillinase regulatory mutants having similar properties have therefore been isolated and characterised in \( B. licheniformis \) and \( S. aureus \). Mutations closely linked to the structural gene \( (p) \), give a magnoco-constitutive phenotype in both systems. Mutations giving a basal level of penicillinase and defective in inducibility can map in genes other than \( i \) \( (i_R \) in \( S. aureus \)); this is indicated by mapping data in \( B. licheniformis \) and from complementation studies in \( S. aureus \). There is no evidence to suggest that any of the mutants isolated in \( S. aureus \) with this phenotype map in \( i \), \( (i.e. \ i_R; \ defined \ by \ mutations \ conferring \ magnoco-constitutivity) \), whereas in \( B. licheniformis \), one base-constitutive mutation \( (i_{28}) \) probably maps in \( i \). If \( i_{28} \) is analogous to \( i^8 \) mutations in the \( lac \) system, it would be expected to be trans-dominant in the heterozygous diploid state; no such mutants have been characterized in \( S. aureus \).

No low level penicillinase mutants (isolated after mutagenesis of a \( pen^{+} \) strain) comparable to those obtained in \( S. aureus \) have been characterized in \( B. licheniformis \), though mutants producing low levels of penicillinase have been isolated (Collins, personal communication); the nature of these mutants has not been studied.
7. Penicillinase Regulation: A Unitary Hypothesis

a) Introduction

Using the genetic evidence presented in this thesis, along with that from the staphylococcal system and the recent biochemical results of Davies (1969) and Imsande (1969), it is possible to formulate a model for the regulation of penicillinase synthesis in Gram-positive bacteria.

b) Biochemical data

Inducer binds specifically and irreversibly at a site associated with the cell membrane, though the cell wall is required for inducer binding (Duerksen, 1964; Imsande, 1969). Approximately 1000 molecules of penicillin can be bound to cells of *B. cereus* (Imsande, 1969) and about 80% of these are retained by protoplasts. It is not known what percentage of these molecules are actually involved in the induction process. The penicillinase mRNA half-life for *B. licheniformis* is 4.7 min. at 37°C. (Davies, 1969), while that for *B. cereus* is about 2 min. at 37°C. (Davies and Imsande, unpublished); these values are less than those suggested by some earlier work (Pollock, 1963; Yudkin, 1966).

After the addition of inducer to cells inducible for penicillinase, the maximum differential rate of penicillinase synthesis is not attained until about one generation time later in *B. licheniformis* and *B. cereus* (Davies, 1969; Imsande, 1969). However a differential synthesis of penicillinase can be measured much sooner (after 30 sec. in *B. cereus*; Imsande, 1969; and after about 12 min. in *B. licheniformis*; Davies, 1969).

As there is no evidence that penicillins actually enter the cell (Duerksen, 1964; Rogers, 1967), one is therefore faced with the problem of how inducer, which binds irreversibly to the cell, causes a maximum differential rate of penicillinase synthesis some 30-50 min. later, presumably by differentially increasing the rate of penicillinase mRNA
A model to explain these results and the genetic data presented in this thesis is now presented.

c) A model for penicillinase regulation

A scheme for penicillinase regulation is shown in Figure 6.

Induction of penicillinase is controlled negatively by a protein repressor (R), the gene product of i (i_R in S. aureus), which is closely linked to the penicillinase structural gene (p). R alone, or in combination with some other element, binds to the penicillinase operator (o), which lies on the i-distal side of p in B. licheniformis. The binding of R to o prevents penicillinase synthesis at the transcriptional level. Mutations in i can give either an increased level of penicillinase (impaired binding of R to o; e.g. i_0, i_09 and i_99 in B. licheniformis) or a basal uninducible penicillinase level (normal binding of R to o and loss of affinity of R for inducing element, which normally prevents binding to o in the presence of inducer; e.g. i_26 in B. licheniformis).

Inducer binds specifically at a site (N) which is associated with the cell-membrane. Components of this site are coded for by at least two genes in B. licheniformis; one of these genes (n!) is about 50% linked to i and p, and contains x_27 and x_14, while the other gene (n!!), unlinked to p and i, contains x_31.

N alone has no affinity for the repressor R, though in the presence of inducer (I), N has a high affinity for R (greater than that of o for R). Since inducer is restrained at the cell-membrane and the operator is located within the cell, N/I can only interact with R when it is free in the cytoplasm, uncomplexed to o. This is in contrast to the lac system, where inducer interacts with repressor on the operator and dissociates the repressor/operator complex.
Other mutations in \( i \), giving an altered \( R \), can give phenotypes other than magno-constitutive and baseo-constitutive. For example, \( R \) showing impaired binding to \( O \) and loss of binding to \( N/I \), would give a baseo-constitutive phenotype, while binding of \( N \) by \( R \), in the absence of \( I \), would give a semi-constitutive phenotype.

Mutations in \( n^1 \) or \( n^2 \) giving altered \( N \), can give either a basal penicillinase level and defective inducibility (e.g. \( x_{27}, x_{14} \) and \( x_{31} \); \( N \) impaired in binding to \( I \), or inability of \( N/I \) to bind \( R \) normally), or a raised level of penicillinase (\( N \) binds \( R \) in absence of \( I \)); mutants of this last type have not been characterized in \( B.licheniformis \) though the \( N2 \) mutants of \( S.eurum \) might be of this type.

The slow differential rate of penicillinase synthesis observed after induction can be explained by the fact that \( N/I \) can only bind \( R \) which is uncomplexed to \( O \). One must also postulate that \( R \) has a relatively long half-life on \( O \). It can be calculated that \( lae \) repressor has a half-life of about 28 min. on \( lae \) operator (calculated from the data of Riggs and Bourgeois, 1969; \( k_b \) for the dissociation of the repressor/operator complex is \( 4 \times 10^{-4} \sec^{-1} \)).

If in the penicillinase system the \( R/O \) complex has a comparable half-life, then it is probable that the observed induction kinetics are the result of the gradual titration by \( N/I \) of free \( R \), resulting from dissociation of \( R/O \) and \( de novo \) synthesis. There are a large number of other parameters which will be responsible for determining the observed induction and repression kinetics. Some of these are: the relative affinities of \( R \) for \( O \), \( R \) for \( N \), and \( R \) for \( N/I \); the relative numbers of \( N \) compared to \( O \); the stability and rate of synthesis of \( R \) and \( N \). Though it is not possible to make estimates of most of these parameters, it seems reasonable to conclude that the model presented is compatible with all the known genetic and biochemical data.
Gene \( p \) is the structural gene for penicillinase and \( O \) is the penicillinase operator. Gene \( I \) synthesizes repressor (R) which binds \( O \) and prevents a high rate of \( p \) transcription. \( N \), the inducer binding site, is associated with the cell-membrane and alone has a low affinity for R (less than that of \( O \) for R). \( N \) is the gene-product of at least two genes (\( n' \) and \( n'' \)).

On induction, inducer (I) binds \( N \) to give \( N/I \) which has a higher affinity than \( O \) for R. As \( N/I \) is restrained at the membrane and \( O \) is located within the cell, \( N/I \) can only bind free R which is either released from \( O \) or synthesized de novo. As R is titrated by \( N/I \), none is available to bind \( O \) and a high rate of transcription of \( p \) occurs.

Gene \( n' \) is about 50% linked to \( i \) and \( p \), whereas \( n'' \) is unlinked. Characterised mutations are shown in their respective genes. Pen \( r_{32} \) (in \( O \)) may be a deletion fusing the penicillinase operon onto the control of another operon (see Discussion: 5).
Figure 6. A Model for the Regulation of Penicillinase Synthesis

(i) Uninduced cell

membrane

basal level of penicillinase

(ii) Induced cell

membrane

induced level of penicillinase

N' + N'' → N
Experiments can now be designed to test the model.

Operationally the only difference between this system and lac is that inducer and operator binding sites of the lac repressor have become functionally distinct units in the penicillinase system: this has been necessitated by the inability of inducer to enter the neighbourhood of the operator region.

A similar model to that presented here for the regulation of penicillinase synthesis, might be applicable to other control systems where substrate (i.e. effector too) cannot enter the cell; consequently the enzyme is found associated with the cell-surface (i.e. cell-wall or membrane) and effector binding sites might be found associated with the cell-surface. In the alkaline phosphatase system in E. coli (Introduction this thesis; Torriani, 1960; Garen and Otsuji, 1964), the alkaline phosphatase enzyme is found associated with the cell-membrane where it is accessible to substrate outside the cell. There are at least two regulatory genes controlling alkaline phosphatase synthesis, one of which is unlinked to the regulatory gene. It would seem possible that one of these genes might synthesize a component for effector binding on the cell-surface.

In conclusion, the investigation of penicillinase regulation at the genetic level has provided results that have allowed a model of penicillinase regulation to be presented. This model may be applicable to other control systems in which there is a permeability barrier to substrate and specific effector. The characterization of the transformation system and of a large number of penicillinase mutants should allow further genetic and biochemical studies to be made in B. licheniformis.
Appendix I

a) Structure of penicillins

\[ R \quad HN \quad \text{CH}_3 \quad \text{CH}_3 \quad \text{COOH} \]

\[ R = \text{aryl} - \text{CH}_2 - \text{CO} - \]

for benzylpenicillin

b) Structure of cephalosporins

\[ R \quad NH \quad \text{CH} \quad \text{COOH} \]

(i) Cephalosporin C

\[ R = \text{CH} \cdot \text{(CH}_2\text{)}_3 \cdot \text{CO} \quad R' = \text{-OOC.CH}_3 \]

(ii) 7-Amino-cephalosporanic acid (7-ACA)

\[ R = \text{H} \quad R' = \text{-OOC.CH}_3 \]
Appendix II. The reaction catalysed by penicillinase.

\[
\begin{align*}
\text{R} & - \text{HN} - \\
\text{S} & - \text{CH}_3 - \\
\text{O} & - \text{N} - \text{COOH}
\end{align*}
\]

\[\xrightarrow{H_2O}\]

\[
\begin{align*}
\text{R} & - \text{HN} - \\
\text{S} & - \text{CH}_3 - \\
\text{HOOC} & - \text{HN} - \text{COOH}
\end{align*}
\]

penicillin \hspace{1cm} \text{penicilloic acid}
Appendix III a. The genetic map of *B. licheniformis* compiled from the results in this thesis and the marker frequency analysis of Tyeryar et al. (1968).
Appendix III b. Genetic map of *B. subtilis* (from Dubnau et al., 1967).
Appendix III c. The genetic map of *E. coli* (from Taylor and Trotter, 1967).
Variable Gene Linkage and the Phased Appearance of Transformants in Bacillus licheniformis. By D. J. Sherratt and J. F. Collins (Department of Molecular Biology, University of Edinburgh, Edinburgh)

The genetic transformation system in Bacillus licheniformis strain ATCC 9945A (Thorne, C. B. & Stull, H. B. (1966), J. Bact. 91, 1012) has been used to transform the structural and regulatory genes for penicillinase, as well as nutritional and antibiotic resistance markers. Competent cultures were obtained by diluting a stationary-state overnight broth culture into a minimal medium. Experiments designed to show the development of competence in the minimal medium revealed that maximal competence for different markers in a multiple auxotroph occurred at different times.

For example, using a strain requiring both adenine (ade-) and isoleucine and valine (ilv-), the ability to produce ade+ transformants was greatest immediately after the dilution, but decreased by 90% during the next hour. The ability to produce ilv+ transformants reached its maximum only after 3½ hr. incubation in the minimal medium. Both the structural and regulatory genes for penicillinase could be co-transformed with this ilv+ gene, but it was observed that the degree of linkage shown by the penicillinase genes to the ilv+ gene varied from > 50% at the time of maximum production of ilv+ transformants, to < 10% at other times.

The experimental evidence points to a phased behaviour of the culture leading to the sequential appearance of transformants, the transforming DNA repairing a unique position on the replicating genome at any given time. Further, DNA added to the culture at the time of maximal competence is integrated in larger pieces than DNA added at a time of poor competence; the latter DNA appears to be degraded into small pieces and largely rendered ineffective for the production of transformants during the time that elapses before the cells can repair the marker under study.
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a. Fate of transforming DNA in *B. subtilis*. J. Bact., 92, 1250.


