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The Genetics of *Bacillus licheniformis* Penicillinase: a Preliminary Analysis from Studies on Mutation and Inter-strain and Intra-strain Transformations

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SUMMARY

A system of transformation in *Bacillus licheniformis* is described, in which a wide variety of markers can be transferred from one strain to another. A number of mutants affecting the regulatory, enzymic and serological aspects of penicillinase production are described, classified and discussed. A control gene for penicillinase synthesis was found to be linked to the structural gene in four wild-type strains of *B. licheniformis*. The electrophoretic properties of the penicillinases produced by the progeny of inter-strain crosses appeared to be affected by the type of cell in which the relevant structural gene was expressed.

INTRODUCTION

This paper presents part of a biochemical, serological and genetic study of bacterial penicillinases currently under way in this laboratory. It is based on a number of inter-strain and intra-strain crosses in *Bacillus licheniformis* which involve penicillinase production, studied by means of transformation with DNA. The phenotypic properties of various penicillinase mutants used and of the transformants obtained are also described and discussed. Since the work was completed Gwinn & Thorne (1964) have reported the transformation of several nutritional markers in a strain of *B. licheniformis*, and Leonard, Mattheis, Mattheis & Housewright (1964) have studied transformation to prototrophy and polyglutamic acid synthesis in this species.

METHODS

Organisms. The parent of all the mutant strains used for transformation studies was *Bacillus licheniformis* 749/1, derived from strain 749 by 'training' it to grow rapidly in 'minimal' medium having NH_4^+ as sole source of N. Strain 749/110 (subsequently referred to as 110) is a streptomycin-resistant and histidine-requiring derivative of 749/1. Strains 749/48 and 749/81 are streptomycin-resistant and methionine-requiring mutants, respectively, also derived from 749/1. These three strains were obtained from Dr P. H. A. Sneath. Strain 246 was received from Dr I. Takahashi (Canadian Department of Agriculture, Ottowa). Strain IRC-1 was

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received from the Institute of Research, Brussels, Belgium. As with strain 749 and its derivatives, strain 6346 was previously referred to as *B. subtilis* (NCTC 6346) (see Pollock, 1965). All of these strains are penicillinase-inducible.

Media. The minimal (glucose, ammonium, salts) medium used was that of Anagnostopoulos & Spizizen (1961). Andrade agar (Kogut, Pollock & Tridgell, 1956) was used as a standard solid nutrient medium and for detecting the production of penicillinase on solid medium. Cephalosporin C (5 μg./ml.) was included in this medium as an inducer when required. Difco Penassay broth was used as a standard liquid nutrient medium. Enzyme preparations for starch electrophoresis and immunological studies were obtained from organisms grown in CHS medium (Collins, 1964). Transformation medium (TM) consisted of the salt mixture used by Anagnostopoulos & Spizizen (1961) + 0·5% glucose, 0·0075% Casamino acids, 0·02% yeast extract, L-histidine (50 μg./ml.), 0·2% additional sodium citrate and 0·005 ml. each of stock iron solution and oligodynamic mixture (Pollock & Kramer, 1958) per 100 ml. medium. Solid medium contained 2% Difco agar. L-Histidine was added to solid media to a concentration of 50 μg./ml., and streptomycin to 1 mg./ml.

Mutagenic treatment. Ultraviolet radiation. Two ml. of distilled water containing about 3×10^6 spores/ml. were irradiated in a Petri dish for 25 sec. at a distance of 50 cm. with a 300 watt u.v. lamp (Hanovia, Slough). The survival after this treatment was about 1%. The irradiated spores were diluted 30 times with Penassay broth and incubated overnight.

Ethylmethane sulphonate (EMS) as mutagen. Spores were suspended in 2 ml. of 0·1 M-sodium phosphate buffer (pH 7·0) containing 0·1 ml. EMS. The suspension was incubated for 20 min. at 35°, washed once with phosphate buffer, diluted 30 times into Penassay broth and incubated overnight.

N-Methyl-N'-nitro-N-nitrosoguanidine (NG) as mutagen. An overnight culture in Penassay broth was centrifuged and resuspended to original volume in 0·2 M-sodium acetate buffer (pH 5·0). To 0·5 ml. of this suspension was added 0·12 ml. of a solution of NG (4 mg./ml.) in acetate buffer. The suspension was incubated, without shaking, for 210 min. at 35° and then diluted 30 times in Penassay broth, followed by overnight growth. This method is essentially that used by A. Garen (personal communication).

Selection of penicillinase mutants. Constitutive mutants were detected by plating about 10^3 colony-forming units in a 2 ml. layer of Andrade agar on a previously poured Andrade plate. The bacteria were then covered with an additional 2 ml. of Andrade agar. After overnight incubation, the plates were flooded with a solution containing about 500,000 units benzylpenicillin. Colonies of penicillinase-constitutive mutants produced pink zones after a few minutes, and were easily picked and purified.

Mutants producing low amounts of enzyme activity were detected by plating 500–1000 constitutive colony-forming units as described above. After overnight incubation at room temperature, the plates were incubated for 4 hr at 50°. The resulting colonies were then 'developed' for penicillinase by the method of Novick (1963), modified by J. F. Collins and M. H. Richmond (personal communication), as follows. A saturated solution of *N*-phenyl-1-naphthylamine-azo-*o*-carboxybenzene (PNAC) was prepared in dimethylformamide. This was mixed with 10^{-2}

M-phosphate buffer (pH 7·0) to a final dimethylformamide concentration of 30 %, allowed to cool for several hours and then filtered. This preparation was poured on to the Petri dishes to be tested and allowed to soak in for about 1 hr. The excess PNAC was rinsed off with distilled water and 10 ml. of 10^{-2} M-phosphate buffer (pH 7·0) containing 300 mg. benzylpenicillin poured on the dish. Within a few minutes the constitutive parent colonies stained a deep purple. Colonies which remained pale were picked with a Pasteur pipette into Penassay broth containing 100 units sterile penicillinase/ml., to destroy excess penicillin. After overnight incubation, the mutant strains were purified and tested.

Transformation procedure. An entire single colony of a competent strain was picked from a minimal medium plate and inoculated into 3 ml. of transformation medium (TM) in a large culture tube. No attempt was made to disperse the organisms. The tube was then incubated on a reciprocal shaker for 20 hr at 35°. At this time 0·15 ml. was removed and added to 1·35 ml. of TM containing 7×10^{-3} M-NaCl. Deoxyribonucleic acid (DNA) prepared from the donor strain was added to the desired concentration (usually 1 μg./ml.). The suspension was incubated and vigorously aerated through a glass tube in a water bath at 40° for 1–2 hr. During this time little or no change in viable count occurred. Deoxyribonuclease (DNAse) was added to a final concentration of 20 μg./ml. along with 10^{-2} M-$MgSO_4$. After 10 min. the culture was plated as desired in order to select transformant clones. The viable count at this time was about 5×10^8/ml.

'$A \rightarrow B$' signifies a cross in which DNA from A (the donor) is used to transform B (the recipient).

Selection of transformants. Transformants to prototrophy were selected by plating on the appropriate minimal medium. Streptomycin-resistant transformants were selected by plating in a layer of agar on a previously prepared bed of Andrade agar. After a 2-hr delay at 35° a third layer of agar containing streptomycin was added. The final streptomycin concentration attained after uniform diffusion throughout the plate was 1 mg./ml.

Penicillinase-producing transformants were selected as follows. Molten Andrade agar (25 ml.) containing about 3×10^6 colony-forming units was pipetted into each Petri dish. Benzylpenicillin was added with the bacteria, to reach a final concentration of 0·03 units/ml. in half the plates and 0·04 units/ml. in the rest. Both concentrations were used in each experiment since the optimal selection concentration was extremely critical and somewhat variable. A top layer of 5 ml. 1·5 % (w/v) Difco agar was poured when the bottom layer had set. The plates were then incubated at 35° for 18 hr. At this time large clusters of minute satellite colonies were visible, often with a larger transformant colony in the centre. Each plate then received $2–10 \times 10^4$ units benzylpenicillin in 1 ml. M-sodium phosphate buffer (pH 7·0). After 20–48 hr at 35°, the satellite growth had lysed and the transformant colonies could be easily picked and purified. Reconstruction experiments showed that, with the recipient strain used throughout these experiments, recovery of both inducible and constitutive donor types was 90–100 %. Transformant colonies were picked with a needle and purified on appropriate media before testing.

Penicillinase assay. Penicillinase was assayed by the method of Perret (1954) and expressed as units (μmoles benzylpenicillin hydrolysed/hr at 30°; Pollock &

Torriani, 1953). Benzylpenicillin, methicillin and cephalosporin C were used as substrates at concentrations of 2·4, 2·4 and 3·0 mg./ml., respectively.

When cephalosporin C was used as a substrate, 20 min. were allowed for the hydrolysis product to react with iodine (on the suggestion of J. F. Collins, personal communication) rather than the usual 10 min. used with benzylpenicilloic acid. A correction factor of 2 was applied, since it is reported that the product (presumed to be cephalosporoic acid) reacts with only 4 equivalents of iodine (Fleming, Goldner & Glass, 1963) as compared with 8 in the case of benzylpenicilloic acid. It should, however, be noted that values for 'cephalosporinase' activity thus assayed are about 30% below those obtained manometrically (Pollock, 1965).

Induction tests: 'induction ratio'. Overnight cultures in Penassay broth were diluted tenfold in the same medium and incubated at 35° with shaking. When an opacity equivalent to a bacterial concentration of 0·1 mg. dry wt. bacteria/ml. (measured by reference to a standard opacity/dry wt. curve) was reached, each culture was divided into two portions, one of which received 2 μg. cephalosporin C/ml. Penicillinase values were measured after further incubation for 1·5 hr and expressed in units/mg. dry wt. bacteria (specific activity). The specific activity of the induced cultures divided by that of the uninduced is referred to as the 'induction ratio'. It should be noted that Induction Ratios are only really valid for comparative purposes under standard conditions, and do not measure the maximum possible extent of induction in any particular case; this would require much longer incubation with inducer.

Preparation of DNA. DNA was prepared by the method of Marmur (1961) and estimated by the method of Burton (1956).

Immunological analysis. Enzyme/antiserum precipitation reactions in agar were detected, and enzyme samples quantitatively titrated, by means of antiserum to wild-type *B. licheniformis* strain 749/C penicillinase, according to methods described elsewhere (Pollock, 1964).

Starch-gel electrophoresis. Horizontal starch-gel electrophoresis was done with penicillinase samples soaked on filter paper, according to the method of Smithies (1955), using gel prepared in 5×10^{-2} M-glycine buffer (pH 8·9). A voltage gradient of about 10 V./cm. was applied at room temperature for 4 hr. The starch blocks were sliced, and penicillinase bands developed on one half by using PNAC and on the other by spraying with 2×10^{-2} M-iodine containing 100,000 units benzylpenicillin/ml. The latter method was more sensitive but more transitory than the first. The resulting patterns were photographed.

Reagents. N-Phenyl-1-naphthylamine-azo-*o*-carboxybenzene was bought from The British Drug Houses Ltd., Poole, England, the ethylmethane sulphonate from Eastman Kodak Co. and the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine from The Aldrich Chemical Co. Methicillin and cephalosporin C were gifts from Beecham Research Laboratories Ltd. and Glaxo Laboratories, respectively.

RESULTS

Throughout this work derivatives from *Bacillus licheniformis* strain 749 were used as recipients.

Table 1 shows that pre-treatment of DNA with DNAse (20 μg./ml.) for 10 min.

completely prevented transformation. DNA prepared from a histidine auxotroph (strain 110) could transform to methionine prototrophy but did not transform strain 110 to independence of histidine. The same 110 culture was competent since it was transformed to prototrophy by wild-type DNA. It proved possible to transform cells of various mutant strains of *B. licheniformis* 749 routinely at a frequency of 10^{-5} to 10^{-6}. All markers tested have been successfully transformed, including streptomycin resistance, histidine, methionine, adenine and arginine prototrophy, and penicillinase production.

When *Bacillus licheniformis* 749-type cultures were plated on minimal medium, two morphological colony types were apparent. The majority were flat, smooth in texture, sticky and soft. A small and variable minority (0·1–10 %) were smaller, rough, often crinkled, and turned reddish brown after 2–3 days. The predominant colony type will be referred to as 'smooth' and the other as 'rough'. In a typical

Table 1. *Strains, markers and transformation frequencies in Bacillus licheniformis*

Strain from which DNA was isolated	Recipient	Selected marker	No. of transformant-type colonies appearing after treatment with DNA
749/48	749/81	Strept. -res.	1930
749/48 (preparation pre-treated with DNAse)	749/81	Strept. -res.	10
749/48	110	*his*$^+$	1850
110	110	*his*$^+$	10
110	749/81	*met*$^+$	2240
None	749/81	*met*$^+$	80
None	749/81	Strept. -res.	15
None	110	*his*$^+$	0

experiment in which 2 smooth and 5 rough variants from strain 110 were transformed to histidine independence, the smooth variants gave 10 and 17 prototrophs/ml. and the rough gave 2540, 3970, 286, 473 and 1430 prototrophs/ml., respectively. When a mixture of rough and smooth variants was used as recipient, all of the colonies selected for histidine or methionine independence were rough. The viable count plates contained about 80 % smooth colonies. It is apparent that the smooth variants were not competent under the conditions used. When rough variants were streaked on minimal medium, reversion to the smooth state was easily demonstrated. The rate of reversion varied markedly among rough strains. Competent rough variants were maintained on minimal agar and re-streaked from single colonies about once a week on fresh plates. Single colonies from these streaks were used to initiate competent cultures.

The concentrations of the ingredients in transformation medium (TM) were those experimentally determined to be optimal. These components were required only during the period of growth preceding the addition of DNA. Once the organisms had become competent, they could be diluted into Spizizen salts medium or saline containing 10^{-3} M-$MgSO_4$ and then exposed to DNA without loss of competence, as compared with controls treated in the usual way. In saline without $MgSO_4$, the transforming efficiency was much lower.

Competence was found to reach a maximum during the stationary phase of growth and to decline rapidly after about 3 hr. The yield of transformants increased linearly with increasing DNA concentration, reaching a maximum at about 1 μg. DNA/ml.

Characterization of penicillinases

Mutants. Table 2 presents some properties of the penicillinase mutants obtained from the standard wild-type ('magno-inducible') strains used. In addition to 'magno-constitutive' strains, several loss mutants, some producing immunologically cross-reacting material (CRM) were obtained. Other mutants with abnormally low induction ratios, presumably with regulatory lesions in a control gene, may be described as 'semi-constitutive' (raised basal, normal maximum induction) or 'semi-inducible' (normal basal, subnormal maximal induction). Others were strictly non-inducible (induction ratios of unity), each producing its characteristic level of enzyme with or without inducer, covering a range from around normal basal or below ('micro-constitutive') up to 10% of the maximum ('magno-') constitutive value ('meso-constitutive'). Treatment at 50° for 1 hr caused more than 90% loss of activity in crude culture supernatant fluids from both these mutants, but had no effect on the parent, wild-type enzyme.

Strain 9 behaved like the inducible wild-type at 35°, but was semi-constitutive at 50°, forming at the higher temperature about 500 units penicillinase/mg. dry wt. bacteria in the absence of inducer and the normal fully inducible complement after induction.

Strain 18 (obtained from a constitutive mutant of 100) represents a particular type of enzyme mutant of which strains 19, 71 and 20 later proved (Pollock, Fleming & Petrie, 1965) to be further examples. In Penassay broth, the total enzyme activity of strain 18 was only about 5–10% of that produced by the parent strain. When the bacteria were lysed with lysozyme, about 50% or more of the penicillinase activity was lost, whereas the low activity in the supernatant fluid was unaffected by lysozyme. When a constant amount of supernatant fluid enzyme was titrated with increasing amounts of specific antiserum to *B. licheniformis* 749/C penicillinase, a three- to four-fold stimulation of penicillinase activity was observed. Under similar conditions wild-type enzyme was 50% inhibited by this antiserum (Pollock, 1964).

Strains 22 and 72 produced no enzyme activity detectable by the methods used; these strains were derived from constitutive strains and produced cross-reacting material (CRM) in the absence of inducer. They were thus double penicillinase mutants, with damage presumably in both the control and the structural genes. Strain 22 was used throughout this work as a recipient in crosses involving selection for penicillinase.

Homologous crosses. The term 'homologous' is used here to describe a cross in which the donor and recipient strains were derivatives of the same wild-type isolate. When the donor and recipient were derived from distinct wild-type strains, the cross is described as 'heterologous'. Strain 749/C3 (a constitutive mutant strain) was transformed to prototrophy by using DNA from the wild-type strain 749. The resulting *his*$^+$ strain (C3/1) was used to transform strain 22. Selection was for penicillinase production and for histidine prototrophy. In this cross 17 penicillinase

Table 2. *Penicillinase properties of strains derived from Bacillus licheniformis strain 749*

Strains Standard	Parent	Mutagen		Penicillinase (units/mg. dry wt. bacteria) Uninduced	Induced	Induction ratio*	Phenotypic description†	Precipitation with specific antiserum to wild-type penicillinase‡
749	749	—		12–24	840–1400	35–117	Magno-inducible (standard wild-type)	+ +
749/1	749	—		12–24	840–1400	35–117	Magno-inducible (trained to minimal medium)	+ +
110	749/1	—		12–24	840–1400	35–117	Magno-inducible (histidine auxotroph: streptomycin-res.)	+ +
Penicillinase mutants								
749/c	749	EMS		3000–5000	3000–5000	1·0	Magno-constitutive (standard constitutive strain)	+ +
749/c2	110	EMS		4530	4750	1·05	Magno-constitutive	n.t.
749/c3	110	EMS		3660	4120	1·02	,,	+ +
749/c4	110	EMS		4190	4320	1·03	,,	n.t.
749/c5	110	EMS		3370	3910	1·16	,,	n.t.
749/c6	110	.		4440	4510	1·02	,,	n.t.
749/c7	110	EMS		5920	5850	0·99	,,	n.t.
749/c8	110	EMS		6970	7430	1·07	,,	n.t.
18	749/c3	UV		280	270	0·95	Meso-constitutive	+
32	110	NG		208	208	1·0	,,	−
19	749/c3	NG		90	90	1·0	,,	+ +
77	749/c	NG		79	77	0·98	,,	−
71	749/c	NG		50	52	1·04	,,	+ +
20	749/c3	NG		33	24	0·7	,,	+ +
17	749/c3	NG		21	25	1·2	Micro-constitutive	−
28	110	NG		24	23	0·95	,,	−
76	749/c	NG		19	25	1·3	,,	+ +
31	110	NG		9	10	1·1	,,	−
75	749/c	NG		3·9	4·2	1·1	,,	+ +
16	749/c3	NG		3·8	2·6	0·7	,,	+ +
74	749/c	NG		2·4	2·2	0·92	,,	−
25	749/c3	NG		2·8	2·1	0·75	,,	−
26	749/c3	NG		0·80	0·90	1·1	,,	−
72	749/c	NG		< 0·05	< 0·05	—	Negative	+ +
22	749/c3	NG		< 0·05	< 0·05	—	,,	+ +
23	749/c3	NG		4250	n.t.	—	(Thermo-labile enzyme)	+
24	749/c3	NG		1420	n.t.	—	,,	+
c1	749/1	EMS		2865	4130	1·44	Semi-constitutive	n.t.
9	749/1	EMS	35°	19	1250	66	Magno-inducible at 35°	+ +
			50°	400	1400	2·8	Semi-constitutive at 50°	n.t.
27	110	NG		15	130	8·6	Semi-inducible	+ +
29	110	NG		21	88	4·1	,,	+
34	110	NG		18	43	2·9	,,	−
30	110	NG		14	31	2·3	,,	−
11	749/1	EMS		12	18	1·5	,,	−

* Induction ratio = differential rate of enzyme synthesis under standard conditions for maximal induction/differential rate of enzyme synthesis without induction. (see Text)

† See text for description of nomenclature.

‡ Based on plate Ouchterlony test on surface growth (in case of inducible strains, after maximal induction) as described by Pollock (1964).

n.t. = not tested.

transformants were picked, and all were found to be constitutive; control platings of strain 22 alone yielded no revertants.

When strain 110/1, an inducible wild-type *his*+ transformant derived from 110, was used to transform strain 22, 89 out of 101 penicillinase transformants tested were inducible; the remaining 12% were constitutive. When a DNA concentration 20 times lower was used, 11 out of 15 transformants (73%) were inducible. These structural and regulatory mutant sites in strain 22 are therefore closely linked by transformation. Two constitutive and 5 inducible transformants from this cross were assayed for penicillinase production, with and without induction. The values of enzyme produced in these transformants were not significantly different from those of the constitutive parent strain of 22 and of the inducible donor strains.

Some crosses were performed with strain 9 (the temperature-sensitive control mutant) as donor and strain 22 as recipient. Transformants were tested on Andrade plates, with and without inducer, at 35° and at 50°. Of 15 transformants tested 6 were constitutive and 9 showed the donor temperature-sensitive character. It was concluded that the regulatory mutation of strain 9 is also linked to the structural gene.

Table 3. *Linkage of structural and control loci for penicillinase in Bacillus licheniformis*

The recipient in all crosses was *B. licheniformis* strain 22 (see Table 2).

Donor strain	Transformants tested (no.)	Inducible (%)	Frequency of histidine and penicillinase transformation*
749/1	101	88	100
IRC-1	37	76	370–610
6346	185	52	46–73
246	45	31	32–43

* These values are percentages of the average transformation frequency obtained in the homologous cross.

Heterologous crosses. A series of crosses was undertaken by using DNA from a number of magno-penicillinase-inducible wild-type *Bacillus licheniformis* strains: 6346, IRC-1-S′ (an EMS-induced mutant of IRC-1, resistant to 1 mg. streptomycin/ml.), 246. In these crosses 20,000 rather than 100,000 units benzylpenicillin/plate were used on the second day of selection, because of the lower resistance of 246 and 6346 to saturating concentrations of benzylpenicillin. Separate experiments showed that the use of 20,000 rather than 100,000 units in homologous crosses with strain 749 would not have affected the earlier results obtained. This permits comparison of results derived from the homologous and heterologous crosses.

Table 3 shows the numbers of inducible and constitutive transformants and the relative transformation frequencies obtained for histidine and penicillinase in the various crosses. Throughout this work the frequency of histidine and penicillinase transformation never differed by more than 50% in a given cross. The last column in Table 3 therefore presents combined data for both these markers. (Neither the strain 22 penicillinase structural marker nor the *his*− marker has ever been observed to revert.) All three heterologous donors could co-transform strain 22 to inducibility

and enzyme function, thus demonstrating linkage of the structural and regulatory loci. The degree of linkage varied from about 80 to 30 %. Table 3 shows that the frequency of linked transformation of the structural and regulatory markers varied directly with the frequency of transformation of the histidine and penicillinase markers between heterologous strains.

Properties of penicillinase produced by the progeny of heterologous crosses

An attempt was made to characterize the enzyme produced by transformants from heterologous crosses containing a 749 genome + genetic material from a foreign strain carrying the structural and regulatory determinants for penicillinase. The specific benzylpenicillinase activities of strains 6346 and 246 (which appear to be indistinguishable, judged by all tests so far made) are about sixfold lower than that of strain 749. Indeed, the purified 749/c penicillinase has a molecular activity on benzylpenicillin nearly 6 times that of the purified enzyme from strain 6346/c (a magno-constitutive mutant from strain 6346; Pollock, 1965). On Andrade agar plates ('developed' by the addition of about 2×10^5 units benzylpenicillin, after induction with 5 μg. cephalosporin C/ml.), this difference was clearly recognizable from the zone size of acid production. The penicillinase types of the progeny of a number of heterologous crosses involving 6346 and 246 donors were tested in this way.

Of 185 strain 6346 → strain 22 'hybrids' examined in the first batch, 182 had maximal values of benzylpenicillinase activity similar to that produced by strain 6346. About half of these were constitutive (Table 3). The remaining 3 transformants, however, produced penicillinase at the strain 749 value, and all were constitutive. Repeated control tests never revealed any reversions of strain 22 back to the magno-constitutive phenotype from which it was derived.

Among 145 further strain 6346 → strain 22 hybrids, another 3 transformants (all constitutive) formed penicillinase at the strain 749 value. Finally, out of 45 hybrid strains obtained by transforming strain 22 with DNA from strain 246, 2 produced 749-type enzyme, and both were likewise constitutive. Thus, out of a total of 475 hybrid transformants, 8 (all constitutive) produced recipient-type enzyme, indicating that repair or suppression of the structural gene mutation in strain 22 had been achieved by means of transformation with heterologous DNA.

The validity of the rapid plate test for distinguishing between 6346-type and 749-type penicillinases was confirmed in several cases by measuring the ratios of activity in hydrolysing cephalosporin C and methicillin relative to benzylpenicillin, as described in Methods. (These differ markedly and characteristically in the wild types; Pollock, 1965.) The results for cephalosporin C are shown in Table 4. The classification of a given transformant penicillinase as 6346-like or 749-like on the basis of the plate test was confirmed in all cases by its relative activity on cephalosporin C and in two cases (T21 and T25) on methicillin.

Finally, the apparent identity of the transformant 6346-type penicillinase with the normal wild-type 6346/c enzyme was confirmed in two of these inter-strain hybrids by comparative titrations with anti-749/c penicillinase antiserum (Fig. 1). Wild-type 6346/c and 749/c penicillinases have been shown to differ by 100 % in the extent of maximal stimulation of their methicillinase activities by this antiserum, and by approximately sixfold in their equivalence points, when this is expressed

in terms of the ratio of the minimum amounts of antiserum causing maximal stimulation of the activities of the two types of enzyme respectively (Pollock, 1964). Figure 1 shows that transformants T21 and T25 gave titration curves indistinguishable from those given by normal 6346/c enzyme. In an analogous experiment with benzylpenicillin as substrate, transformants T13 and T33 both gave, with the same antiserum, the 'biphasic' titration curves characteristic of the 6346-type penicillinase (Pollock, 1964).

Table 4. *Bacillus licheniformis: relative activities of wild-type and inter-strain (6346 → 22) transformant penicillinases on different substrates*

Type strains (parental)	Phenotype inferred from benzylpenicillinase plate test	Activity of penicillinase in hydrolysing cephalosporin C (as % activity on benzylpenicillin)
6346/c	6346 constitutive	10·3
749/c3	749 constitutive	0·76
Inter-strain transformants tested		
T17	6346 inducible	10·4
T18	6346 inducible	9·6
T20	6346 constitutive	10·3
T21	6346 constitutive	9·0
T22	6346 constitutive	10·5
T23	6346 constitutive	10·0
T16	749 constitutive	0·73
T25	6346 constitutive	9·7
T27	6346 constitutive	9·5
T28	6346 constitutive	10·0
T30	6346 constitutive	9·3
T31	6346 inducible	9·4
T33	6346 constitutive	9·0
v-3	749 constitutive	0·78
v-4	749 constitutive	0·83

In spite of the fact that all the strain 6346 → strain 22 transformants tested produced a type of penicillinase with enzymic and serological properties indistinguishable from one or other of the two wild-types, electrophoretic mobilities of some of these transformant enzyme samples appeared to be significantly different from both. On electrophoresis through starch gel (see Methods), samples of wild-type 6346/c penicillinase always moved more rapidly than the 749/c enzyme, though both types showed multiple banding (Pollock, 1965). Culture supernatant fluids from ten of the strain 6346 → strain 22 transformants which were enzymically 6346-like and from both wild-types were dialysed and concentrated 15-fold by freeze-drying, and mobilities compared by this method. The penicillinase, in preparations from all ten transformants, migrated at speeds intermediate between those of 6346/c and 749/c. All these produced an enzyme-type enzymically similar to that of 6346/c: typical results are presented in Fig. 2. In electrophoretic runs of artificial mixtures of preparations from transformants and 6346/c wild-type strains, each 'brand' of

penicillinase retained its characteristic mobility unaffected by the presence of the other. Two 749-like transformant preparations from strain 6346 → strain 22 crosses migrated at the same speed as the 749/c preparation.

To determine whether transformation of strain 22 to produce active penicillinase led to elimination of the ability to form the mutant penicillinase protein (cross-reacting material: CRM) normally produced by strain 22, culture supernatant fluids from strain 6346 → strain 22 transformants were examined for the presence of material interfering with the normal penicillinase/antipenicillinase reaction. Interference by the enzymically inactive strain 22 mutant-penicillinase CRM itself was

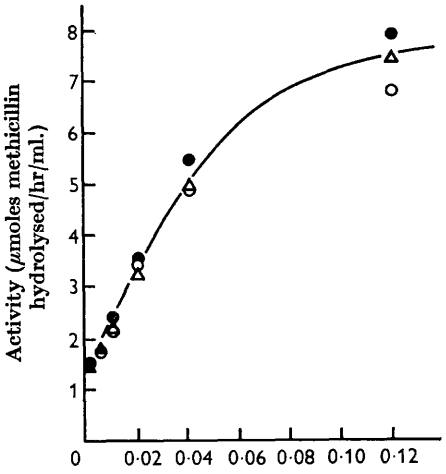

Fig. 1

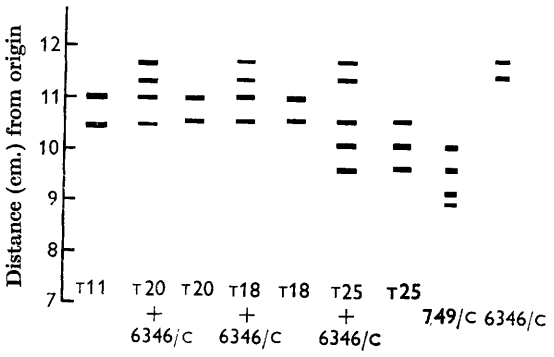

Fig. 2

Fig. 1. Immunological titration curves of penicillinase from 6346/c (wild-type) and 6346→ 22 transformants. Increasing quantities of anti-749/c penicillinase antiserum were added to a constant amount (150 units) of enzyme from untreated culture supernatants, and the resulting enzyme activities determined with methicillin as substrate. —●— 6346/c control; —△— T21; —○— T25.

Fig. 2. Tracings of patterns of enzyme reaction bands after starch gel electrophoresis of inter-strain transformant (T11, T18, T20 and T25) and parental-type (749/c and 6346/c) penicillinases (singly and together). Migration is toward the anode. See text for origin and relationship of enzyme types.

shown by measuring the stimulating effect of increasing quantities of antiserum to 749/c penicillinase on the rate of methicillin hydrolysis by normal (strain 6346) and transformant (strain T25) enzyme, as follows. 100 units of normal enzyme contained in 0·45 ml. supernatant fluid from cultures of strains 6346/c and T25, grown to a population density equiv. to 1·2 mg. dry wt. bacteria/ml., were titrated (*a*) alone and (*b*) after addition of an equivalent quantity (0·5 ml. of culture supernatant fluid from mutant 22 grown to an opacity equiv. to 1·1 mg. dry wt. bacteria/ml.) of the CRM penicillinase. The titration curves obtained (Fig. 3) show (*a*) significant (up to 50 %) inhibition, by an equivalent amount of strain 22 CRM penicillinase, of the reaction between antiserum and both wild-type and transformant enzymes, indicating nearly equal competition between CRM-22 and wild-type enzyme for combination with limiting quantities of antibody; (*b*) no significant titration differences between

normal 6346-type enzyme and that formed by T 25 (as previously indicated in Fig. 1). Similar results were obtained with another strain 6346 → strain 22 transformant, T21. If either of these two transformants had formed significant quantities of 22 CRM penicillinase as well as normal 6346-type enzyme, their enzyme titration curves with antiserum would have been different from that of the 6346/c enzyme alone.

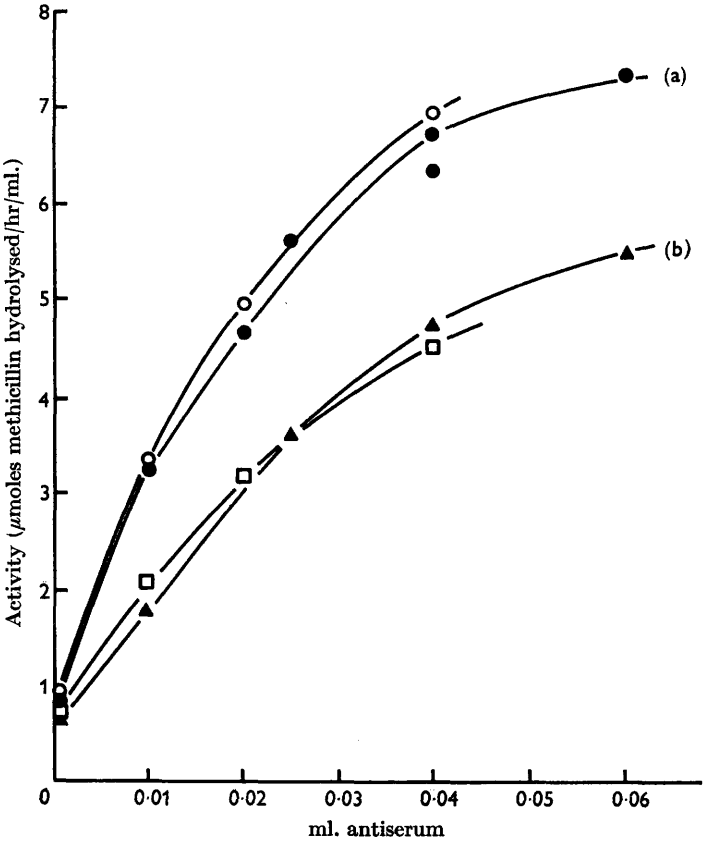

Fig. 3. Interference in the penicillinase/antipenicillinase reaction through addition of enzymically inactive CRM penicillinase from mutant 22. Titration curves showing comparative stimulating effects of increasing quantities of anti-749/c penicillinase antiserum on rates of methicillin hydrolysis by 100 units of wild-type (6346/c) and transformant (T25) enzyme (*a*) alone, and (*b*) after addition of equivalent amount of 22 CRM penicillinase. 6346/c alone —●—●—, T25 alone —○—○—, 6346/c+22 CRM —▲—▲—, T 25+22 CRM —□—□—.

DISCUSSION

The various penicillinase mutants listed in Table 2 can be provisionally grouped into two main categories.

'*Control*' *mutants*, which produce penicillinase qualitatively indistinguishable from the 'magno-inducible' wild-type, but at a significantly different rate or with an altered induction response. These comprise: (i) strains 749/c to 749/c8 inclusive (see Table 2), which form maximal amounts of enzyme in the absence of inducer

and conform to the original definition of 'magno-constitutive' (Pollock, 1957); (ii) strains 27, 29, 30 and 11 (all 'semi-inducible'), which retain some response to induction but form less enzyme than the wild-type, and strain c1 ('semi-constitutive'), which has a significantly increased basal (uninduced) value; both these subgroups have, of course, subnormal induction ratios; (iii) 'micro-constitutives' and 'meso-constitutives', which show no induction response but form enzyme at rates well below the maximum; some of these (77, 17, 74, 25 and 26) arise from strains already mutated to magno-constitutivity, whereas others (28, 31 and 32) stem directly from the original inducible strain 110; (iv) Strain 9, a 'facultative' constitutive, inducible at 35° but semi-constitutive at 50°, having properties consistent with the presence of a thermo-labile repressor, and very similar to the *Escherichia coli* β-galactosidase mutant described by Horiuchi & Novick (1961).

Structural gene mutants, which form enzyme with properties clearly differentiated from those of the wild-type—having either markedly decreased specific activity (as indicated by the presence of a normal serological precipitation reaction, combined with greatly decreased enzyme activity: strains 18, 19, 71, 20, 76, 75, 16, 72 and 22) or decreased thermostability (strains 23 and 24). Strains 18, 19, 71 and 20 show altered serological behaviour, wild-type activity being partially or completely restored after interaction with specific antiserum. One explanation is that this is due to partial restoration of the original wild-type conformation by combination with antiserum, it being supposed that the mutated enzyme in solution adopts an altered conformation which is enzymically inefficient (see Pollock *et al.*, 1965).

Novick (1963) reported on a genetic analysis by transduction of the penicillinase of *Staphylococcus aureus*, and presented evidence indicating that the penicillinase gene of that organism was carried on a plasmid. In *Bacillus licheniformis*, however, there is no suggestion that the penicillinase genes are extra-chromosomal; the structural gene is stable and, unlike the situation in *S. aureus*, there is no large class of absolute negative, irreversible, loss mutants.

The data in Table 4 show that the frequency of co-transformation of the structural and control genes in three heterologous crosses was directly proportional to the frequency of histidine and penicillinase transformation. It seems reasonable to interpret these results in terms of the degree of genetic homology between the strains, since homology over a large stretch of chromosome may be required to integrate a more extended region of donor DNA. B. W. Catlin (private communication) has obtained a similar result in transformational crosses between strains of *Neisseria catarrhalis*; the frequency of co-transformation of resistance to streptomycin and kanamycin was higher in homologous than in heterologous strains. This concept is borne out in the present case by what is known about the phenotype of the four strains of *Bacillus licheniformis* involved. The penicillinase of *B. licheniformis* IRC-1 is more closely related in substrate 'profile' and antiserum titration values to that of strain 749 than are the penicillinases produced by strains 246 or 6346. Furthermore, several phages, isolated from soil, form plaques on either strain 749 or strain IRC-1, but not on strain 6346 or 246 (D. A. Dubnau, unpublished observations).

It seems highly probable that the structural penicillinase loci in the wild-type strains 749 and 6346 are strictly allelic. Apart from *a priori* considerations based on the similarity of the two strains and the two types of enzyme, the results of

interstrain (6346 → 22) crosses indicate that (*a*) insertion of the 6346 type of enzyme into a 749-type recipient (22) involves the exclusion of the normal cross-reacting material (CRM) product of the mutant 22 structural gene, and (*b*) in a few, but significant number of, instances apparently complete repair of the defective 749 penicillinase structural gene in strain 22 can be achieved by means of transformation with DNA from the heterologous wild-type strain 6346. The 749 and 6346 types of penicillinase have been shown (Pollock, 1965) to be closely related proteins with similar molecular weights, differing considerably in their enzymic properties but with only marginal distinctions physico-chemically and serologically. Analyses of their overall amino-acid compositions do not show significant differences, and it is not therefore yet certain that their primary amino-acid sequences are dissimilar. It is, however, more likely that they do differ by a number of residues.* If this be so, the most probable explanation of the repair, by strain 6346, of the defect in strain 22 is the occurrence of an intra-cistronic recombination involving the restricted insertion into the 749-type (22) genome of that portion of the 6346 penicillinase structural gene which is identical with, or very similar to, the homologous region on the 749 genome containing the mutational defect expressed in strain 22.

The fact that 100 % (8 out of 8) of these particular transformants are constitutive (i.e. retain the control mutation of the recipient) indicates that crossing-over must have occurred between the control and structural mutated loci in strain 22. If a region of dissimilarity within the 6346 and 749 genes exists between the position of the mutation in strain 22 and the proximal end of the control gene, a strong selection for constitutivity among the 749-like transformants would be expected.

The overwhelming majority of strain 6346 → strain 22 transformants produced a donor (6346)-type penicillinase. This was to be expected, and it is probable that in most of these transformations the 749-type recipient has accepted the 'foreign' heterologous gene, with little or no modification. However, in some instances it is possible that a 6346-type enzyme may also result from intra-cistronic recombination. The relatively high (about 50 %) incidence of constitutives amongst such transformants suggests that, since close linkage between control and structural penicillinase genes has already been demonstrated in homologous crosses, a proportion of recombinations between the control and structural mutated loci in strain 22 may have occurred within the structural gene. If so, they must involve insertion not only of the area defective in strain 22 but of the fragment of the strain 6346 structural penicillinase gene which carries all or most of the sequences differing in the two types. The distinctive electrophoretic behaviour of all of the 6346-like transformants is also consistent with this possibility, although three inducible hybrid transformants tested showed this same behaviour. The fact that differently treated preparations of exo-enzyme from the same wild-type strain can also differ significantly in their mobilities as shown by this technique (e.g. as between a supernatant fluid concentrate and a fully purified sample; Pollock, 1965) suggests that these differences do not involve the primary amino acid sequence and may arise from the influence of a 749-type cytoplasmic environment acting on a 6346-type of penicillinase molecule.

* See Note page 21

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Note added in proof. At least one difference in the amino-acid sequences has now been unequivocally demonstrated by R. P. Ambler (private communication).