Regulation of Synthesis of Benzyl Alcohol Dehydrogenase in *Acinetobacter calcoaceticus* NCIB8250

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Specific activity of benzyl alcohol dehydrogenase in carbon-limited continuous cultures was at a maximum at a specific growth rate of 0.2 h^{-1} , but fell off at lower and higher growth rates. The specific activity in nitrogen-limited cultures was always lower and was inversely proportional to growth rate. There was severe repression of benzyl alcohol dehydrogenase during metabolism of L(+)-mandelate or phenylglyoxylate in batch cultures. Synthesis of benzyl alcohol dehydrogenase was followed in experiments where various compounds, including a gratuitous inducer and an anti-inducer of the mandelate enzymes, were added to uninduced or pre-induced cultures and to constitutive and blocked mutants. The results led to the conclusion that there were at least two types of repression. One was caused by phenylglyoxylate carboxy-lyase (or a compound synthesized co-ordinately with it), but not by the other mandelate enzymes or by L(+)-mandelate, phenylglyoxylate, benzaldehyde or benzoate. A second type of repression was observed during rapid growth or after the addition of compounds such as succinate which are rapidly and completely metabolized.

INTRODUCTION

Acinetobacter calcoaceticus NCIB8250 oxidizes benzyl alcohol and L(+)-mandelate by a converging pathway (Fig. 1). Results from growth experiments suggested that benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II were repressed during metabolism of L(+)-mandelate, benzoate and subsequent intermediates of the common pathway (Cook, Beggs & Fewson, 1975; Beggs, Cook & Fewson, 1976). The results in the present paper, some of which have been reported in a preliminary form (Beggs & Fewson, 1974; Moyes & Fewson, 1976), indicate that benzyl alcohol dehydrogenase is subject to repression by phenylglyoxylate carboxy-lyase (or a compound synthesized co-ordinately with it) and also to catabolite repression.

METHODS

Organisms and growth media. Bacterial strains (Table 1) were maintained and media were prepared as described earlier (Kennedy & Fewson, 1968*a, b*; Livingstone *et al.*, 1972; Cook *et al.*, 1975).

Growth of bacteria. Inocula for experiments to measure kinetics of enzyme induction were grown in 5 mM-L-glutamate/salts medium using a 0.1% inoculum of a 24 h nutrient broth culture. The cultures were grown at 30 °C with vigorous aeration (Harvey, Fewson & Holms, 1968; air flow rate 200 ml min^{-1}). After 13 to 14 h the bacteria had reached the final generation and were harvested (Cook *et al.*, 1975) and washed once by suspension in cold sterile basal medium followed by re-centrifugation. In some experiments the bacteria were pre-adapted in various ways by adding a second carbon source (or gratuitous inducer) during the penultimate generation ($E_{500} = 0.21$ to 0.43). Incubation was continued for a further 40 to 130 min and then the cultures were harvested and washed.

Growth was followed by measuring E_{500} in a Unicam SP800 spectrophotometer (Cook *et al.*, 1975).

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Fig. 1. Metabolism of benzyl alcohol and L(+)-mandelate by *Acinetobacter calcoaceticus*. 'Benzoate oxidase' is probably benzoate oxygenase plus cyclohexadienediolcarboxylate dehydrogenase (Reiner, 1971). L(+)-Mandelate dehydrogenase, phenylglyoxylate carboxy-lyase (benzoylformate decarboxylase; EC 4.1.1.7) and benzaldehyde dehydrogenase I are co-ordinately induced by phenylglyoxylate or gratuitously induced by thiophenoxyacetate (Livingstone & Fewson, 1972); they are known as the R1 (regulon 1; Kennedy & Fewson, 1968*a*) enzymes. Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II are co-ordinately induced by benzyl alcohol or benzaldehyde (Livingstone *et al.*, 1972) and are known as the R2 (regulon 2) enzymes.

Continuous cultures were grown in a chemostat similar to that described by Baker (1968) with a growth volume of about 600 ml. The medium was the same as that used for batch culture but it also contained a mixture of trace metals (2 ml l⁻¹ in the complete medium) prepared as follows. Nitrilotriacetic acid (50 g) was dissolved in 1 M-NaOH (625 ml) and the solution was adjusted to pH 7.0 with 5 M-HCl. Salts were then added in the following order, each being allowed to dissolve before the next was added: FeSO₄·7H₂O, 1.1 g; Na₂MoO₄·2H₂O, 50 mg; MnSO₄·4H₂O, 50 mg; ZnSO₄·7H₂O, 50 mg; CuSO₄·5H₂O, 25 mg; CoCl₂·6H₂O, 25 mg. The solution was made up to 1 l and autoclaved at 109 °C.

Measurement of enzyme induction and repression. Experiments in which enzyme activities were measured after growth for 2 h (e.g. Tables 2, 3 and Fig. 7) were done as described by Livingstone *et al.* (1972).

Kinetics of enzyme induction and repression were followed in cultures growing at 30 °C in appropriate salts media (500 ml) in 1 l flasks fitted with side-arms, using the stirring apparatus described by Harvey *et al.* (1968; air flow rate 200 ml min⁻¹). The inocula, grown as described above, were suspended in basal medium (5 to 10 ml) and gave an initial turbidity (*E*₅₀₀) of approx. 0.4. Samples were taken at intervals to measure growth (Cook *et al.*, 1975). Other samples (10 ml) were removed for enzyme assay: these were transferred to 15 ml polypropylene centrifuge tubes in an ice-water slurry and centrifuged as soon as possible at 15000*g* for 20 min at 4 °C in an MSE 'Highspeed 18' centrifuge using a 6 × 250 ml rotor fitted with rubber adaptors

Table 1. *Strains of Acinetobacter calcoaceticus*

Strain*	Characteristics
Wild type (NCIB8250)	Inducible for R1 and R2 enzymes (Kennedy & Fewson, 1968 <i>a</i>)
NF1408 (NCIB10789)	Lacks L(+)-mandelate dehydrogenase (Livingstone, 1970)
NF0410	Lacks L(+)-mandelate dehydrogenase (Livingstone & Fewson, 1972)
NF1706 (NCIB10788)	Lacks phenylglyoxylate carboxy-lyase (Livingstone & Fewson, 1972)
NA36 (NCIB10791)	Lacks phenylglyoxylate carboxy-lyase (Livingstone & Fewson, 1972)
NF2405	Lacks all R1 enzymes (Livingstone & Fewson, 1972)
NF0113 (NCIB10790)	Lacks all R1 enzymes (Livingstone, 1970)
NA21 (NCIB10792)	Grows on catechol but not on benzoate or on any compound metabolized via benzoate. No detectable decarboxylation of [<i>carboxy</i> - ¹⁴ C]benzoate. Shows very low activities of catechol 1,2-oxygenase in the presence of benzoate. Low rate of O ₂ -uptake with benzoate. Benzoate appears to get into the bacteria. Probably lacks, or is leaky for, at least one component of 'benzoate oxidase'. (A. Livingstone, A. M. Cook & J. D. Beggs, unpublished observations)
HMM5	Constitutive for the R1 enzymes. Grows on benzyl alcohol only after long lag. (Moyes & Fewson, 1976; H. M. Moyes, unpublished observations)
J8	Derived from HMM5 on basis of ability to grow immediately on benzyl alcohol. Lacks L(+)-mandelate dehydrogenase and phenylglyoxylate carboxy-lyase (this paper, Table 3).
J8/R1	Derived from J8 on basis of ability to grow immediately on phenylglyoxylate. Constitutive for R1 enzymes (this paper, Table 3).

Two uncharacterized batches of mutants derived from the wild type and from NF1408, isolated solely on the basis of ability to grow on 2 mM-phenylglyoxylate in the presence of 2 mM-2-phenylpropionate (this paper, Fig. 7).

* All mutants with the prefix N were obtained after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; other mutants arose spontaneously. Except for the second- and third-stage mutants J8 and J8/R1 and the uncharacterized mutants derived from NF1408, all strains were derived directly from the wild type.

holding five tubes each. The supernatant media were discarded and the bacterial pellets were assayed immediately or stored at -20 °C and assayed later.

Measurement of enzyme activities. The general procedures were described by Beggs *et al.* (1976).

For the determination of phenylglyoxylate carboxy-lyase (benzoylformate decarboxylase; EC 4.1.1.7), harvested bacteria were resuspended in ice-cold salts medium to a turbidity of about 1 at 500 nm. A portion of the suspension (2 ml) was added to 10 μ l toluene in a 6 $\times$ 0.75 in test tube and mixed for 30 s on a vortex mixer. The tube, covered with a metal cap, was incubated at 27 °C for 4 h. The treated bacteria were then assayed in reaction mixtures containing: 400 μ mol KH₂PO₄/K₂HPO₄ buffer, pH 7.0; 6 μ mol NAD⁺; 200 nmol thiamin pyrophosphate (dissolved in 0.2 M-KH₂PO₄/K₂HPO₄ buffer, pH 7.0); 0.11 mg aldehyde dehydrogenase (added in 0.1 ml of a solution in 1 mM-dithiothreitol); 15 μ mol freshly prepared phenylglyoxylic acid (adjusted to pH 7.0) to initiate the reaction; total volume 3.0 ml. The rate of NAD⁺ reduction was followed at 340 nm; the decarboxylation of 1 μ mol phenylglyoxylate to benzaldehyde, and the oxidation of the benzaldehyde by the excess aldehyde dehydrogenase, gives a ΔE_{340} of +2.07.

For the determination of benzaldehyde dehydrogenase, harvested bacteria were resuspended in ice-cold salts medium (containing 2 mM-dithiothreitol) to a turbidity at 500 nm of about 4 if the specific activity was less than 50 nmol min⁻¹ (mg protein)⁻¹ or to a turbidity of about 1 if the specific activity was greater than this. A portion of the suspension (2 ml) was added to 0.1 ml 4% (v/v) toluene in ethanol in a 6 $\times$ 0.75 in test tube and mixed for 30 s. The tube was covered with a metal cap and incubated at 27 °C for 4 h. Treated bacteria were assayed in reaction mixtures containing: 200 μ mol Na₂P₂O₇/HCl buffer, pH 9; 6 μ mol NAD⁺; 50 μ mol KCl; 300 nmol freshly prepared benzaldehyde to initiate the reaction; total volume 3.0 ml. The rate of NAD⁺ reduction was followed at 340 nm.

Activities of L-mandelate dehydrogenase and benzyl alcohol dehydrogenase were measured as described by Beggs *et al.* (1976).

Enzyme units are defined as μ mol substrate converted min⁻¹; specific activities are given as munits of enzyme (mg protein)⁻¹.

Differential rates of enzyme synthesis were calculated from the slopes of graphs of [increase in enzyme activity (munits ml⁻¹)] against [increase in bacterial protein (mg ml⁻¹)].

Materials. All reagents were of the best grade that could be obtained commercially and, with the excep-

Fig. 2. Effect of growth rate on the synthesis of benzyl alcohol dehydrogenase. *Acinetobacter calcoaceticus* NCIB8250 was grown in a chemostat under conditions of carbon-limitation [1.5 mM-benzyl alcohol, 1 g (NH₄)₂SO₄ l⁻¹; solid symbols] or nitrogen-limitation [5 mM-benzyl alcohol, 0.08 g (NH₄)₂SO₄ l⁻¹; open symbols]. Each shape of symbol represents results from an independent chemostat run. At least 10 replacement volumes of medium were added before each sample was taken for assay. Replicate values at the same growth rate in a particular chemostat run represent separate samples taken at least 10 replacement volumes apart.

tion of the compounds listed below, were obtained from the sources indicated by Livingstone & Fewson (1972), Livingstone *et al.* (1972), Cook *et al.* (1975) and Beggs *et al.* (1976). Thiophenoxyacetic acid was obtained from Fluka, Buchs, Switzerland; L-glutamic acid.HCl from T. J. Sas & Son, London; aldehyde dehydrogenase grade II (bakers' yeast), cyclic AMP and thiamin pyrophosphate chloride from Sigma; and 2-phenylpropionic acid from Aldrich Chemical Co., Gillingham, Dorset and from K & K, Kodak.

RESULTS

Factors affecting the rate of synthesis of benzyl alcohol dehydrogenase

Growth rate. Continuous culture of *A. calcoaceticus* with benzyl alcohol as limiting carbon source gave a maximum specific activity of benzyl alcohol dehydrogenase [4 units (mg protein)⁻¹] at a specific growth rate of about 0.2 h⁻¹ (Fig. 2). This pattern is quite common for catabolic enzymes (review by Dean, 1972). The decrease in activity at high growth rates may be due to catabolite repression (Clarke, Houldsworth & Lilly, 1968), but the specific activity was not increased by adding 10 mM-cyclic AMP to bacteria growing at growth rates of 0.44 or 0.52 h⁻¹.

Under conditions of nitrogen-limitation, the specific activity of benzyl alcohol dehydrogenase rose gradually with decreasing growth rate and there was substantial repression at intermediate growth rates (Fig. 2).

Batch culture on benzyl alcohol showed a growth lag if the inoculum had not been pre-induced for the R2 enzymes (Beggs *et al.*, 1976). When the inoculum had been pre-induced, growth on benzyl alcohol was exponential (specific growth rate 0.88 h⁻¹) and benzyl alcohol dehydrogenase was initially synthesized at a differential rate of 750 munits (mg protein)⁻¹; this is in good agreement with an extrapolation of Fig. 2. The specific activity declined throughout growth although the concentration of benzyl alcohol was well above that required for maximum induction. This progressive repression complicates analysis of kinetics of enzyme synthesis and is one of the reasons why we followed enzyme induction during growth on an alternative carbon source during the majority of experiments described in this paper.

L-Glutamate as principal carbon and energy source for growth during measurements of kinetics of enzyme induction. Benzyl alcohol dehydrogenase was synthesized at a differential rate of 1500 munits (mg protein)⁻¹ during growth on 5 mM-L-glutamate with 1.5 mM-benzyl alcohol as inducer if the inoculum had been grown on L-glutamate (Fig. 3). The same differential rate was obtained with 2.5 or 10 mM-L-glutamate. Maximum differential

Fig. 3

Fig. 4

Fig. 3. Synthesis of benzyl alcohol dehydrogenase during growth of *A. calcoaceticus* NCIB8250 on L-glutamate. Uninduced inocula (○, ●) were prepared by growth on L-glutamate. Pre-induced inocula (△, ▲) were also grown on L-glutamate but benzyl alcohol (final concn 1.5 mM) was added 60 min before harvesting. These inocula were added to salts media (500 ml) containing 5 mM-L-glutamate (●, ▲) or 5 mM-L-glutamate plus 1.5 mM-benzyl alcohol (○, △). Samples were taken at intervals for measurement of growth (E_{600}) and benzyl alcohol dehydrogenase activity. Values quoted on the graphs are differential rates of enzyme synthesis [munits (mg protein)⁻¹].

Fig. 4. Effect of phenylglyoxylate on induction of benzyl alcohol dehydrogenase by benzyl alcohol. An inoculum of *A. calcoaceticus* NCIB8250 adapted to growth on L-glutamate was added to 5 mM-L-glutamate/salts media (500 ml) containing 1.5 mM-benzyl alcohol (○), 1.5 mM-phenylglyoxylate (△), or 1.5 mM-benzyl alcohol plus 1.5 mM-phenylglyoxylate (□). Samples were taken at intervals for turbidity measurements and benzyl alcohol dehydrogenase assays. Values quoted on the graphs are differential rates of enzyme synthesis [munits (mg protein)⁻¹].

rates of synthesis were achieved after about 15 min; this is rather a long time if only transcription and translation have to achieve maximum rates. In this and subsequent experiments, the differential rate of synthesis decreased after about 1 h. There could be several reasons for this: (i) the rate of growth might increase because of the metabolism of benzyl alcohol, and this would diminish the rate of synthesis (Fig. 2); (ii) metabolites of benzyl alcohol might cause repression; (iii) the concentration of inducer might no longer be sufficient (see Fig. 8). Figure 3 shows that an uninduced inoculum synthesized benzyl alcohol dehydrogenase just as efficiently as a pre-induced inoculum. Under these conditions growth occurred at a rate of about 0.46 h⁻¹ so the glutamate must have caused very little, if any, repression compared with that occurring in the chemostat using benzyl alcohol as sole carbon source at the same growth rate (Fig. 2). Concentrations of benzyl alcohol from 0.2 to 5 mM all gave the same differential rate of synthesis, as did 0.2 mM-benzaldehyde; higher concentrations of benzaldehyde inhibited growth.

With the uninduced inoculum, there was a small burst of synthesis followed by a continuing slow rate of synthesis of benzyl alcohol dehydrogenase [15 munits (mg protein)⁻¹] in the absence of added inducer (Fig. 3). This enzyme has not been shown to be identical with the inducible benzyl alcohol dehydrogenase, but there is no reason to doubt that it is and in many respects it behaves similarly (see later). In subsequent experiments this 'endogenous' rate of synthesis was subtracted from the measured rates in all estimates of degrees of induction and repression. With the pre-induced inoculum, there was some synthesis of benzyl alcohol dehydrogenase after transfer to glutamate/salts medium without benzyl alcohol (Fig. 3); this may represent expression of pre-existing mRNA.

Table 2. *Repressive effects of various compounds on benzyl alcohol dehydrogenase in the wild type and mutant strains of A. calcoaceticus*

Acinetobacter calcoaceticus NCIB8250 and various mutant strains derived from it were grown in salts medium containing 5 mM-L-glutamate plus 2 mM-benzyl alcohol, and in the same medium supplemented with other compounds. After 2 h the bacteria were harvested and benzyl alcohol dehydrogenase activities were measured.

(a) *Effect of L(+)-mandelate and phenylglyoxylate on induction by benzyl alcohol*

Strain	Control benzyl alcohol dehydrogenase activity after growth in presence of benzyl alcohol [munits (mg protein) ⁻¹]	Degree of repression (%) after addition of:	
		L(+)-Mandelate (2 mM)	Phenylglyoxylate (2 mM)
NCIB8250 (wild type)	295	96	95 (94-98)*
NFI408	239	0	100 (90-100)*
NFO410	272	2	100
NFI706	365	NT	11
NA36	385	NT	2 (0-5)*
NF2405	298	NT	0
NFO113	279	NT	0
NA21	823	NT	98 (87-98)*

NT, Not tested.

* Range of values found in other experiments.

(b) *Effect of various compounds on the wild-type strain NCIB8250*

Addition to medium	Degree of repression (%)	Addition to medium	Degree of repression (%)
Benzoate (2 mM)	33	Succinate (2 mM)	75
	(27-34)†	Succinate (5 mM)	100
Benzoate (5 mM)	40	Acetate (2 mM)	52
Catechol (2 mM)	97	Acetate (5 mM)	100
		Thiophenoxyacetate (1.5 mM)	50‡

† Range of values found in other experiments using 1.5 mM-benzoate plus 1.5 mM-benzyl alcohol.

‡ The benzyl alcohol concentration was only 1.5 mM in this experiment.

The results justify our use of L-glutamate as principal carbon and energy source during the later experiments on kinetics of enzyme induction and repression: (i) there was immediate growth on L-glutamate whatever the nature of the inoculum; (ii) there was no significant repression above that inherent in growth on benzyl alcohol at the same growth rate; (iii) inocula pre-induced in various ways could be used; (iv) growth continued whether benzyl alcohol dehydrogenase was induced or repressed; (v) blocked mutants unable to grow on benzyl alcohol could be used.

Repression of benzyl alcohol dehydrogenase

Uninduced inocula. L(+)-Mandelate, phenylglyoxylate, benzoate, catechol, succinate, acetate and thiophenoxyacetate all caused repression of benzyl alcohol dehydrogenase in the wild-type strain (Table 2). Similar results were obtained in experiments using benzaldehyde as inducer. A time-course of the effect of phenylglyoxylate on the induction of benzyl alcohol dehydrogenase by benzyl alcohol is shown in Fig. 4. The differential rate of synthesis was initially repressed by 94% relative to that in the culture without phenylglyoxylate.

Mutants of *A. calcoaceticus* lacking various enzymes of the mandelate pathway were used to investigate the cause of the repression of benzyl alcohol dehydrogenase by L(+)-mandelate and phenylglyoxylate (Table 2). There was no repression by L(+)-mandelate in mutants NFI408 or NFO410 [which lack L(+)-mandelate dehydrogenase]; in these mutants there would be no induction of the other two R1 enzymes since phenylglyoxylate (the inducer) could not be formed (Livingstone & Fewson, 1972). These two mutants, however,

did show repression in the presence of phenylglyoxylate. Mutants NF1706, NA36 (which lack phenylglyoxylate carboxy-lyase), NF2405 and NF0113 (which lack all three R1 enzymes) were not repressed in the presence of phenylglyoxylate. Figure 5(a) shows that there was never even a transient repression for mutant NA36. Mutant NA21 was repressed by phenylglyoxylate (Table 2) and did not escape from repression at any time in a 2 h experiment (Fig. 5b).

Benzaldehyde is an inducer of the R2 enzymes (Livingstone *et al.*, 1972), but an experiment was carried out to investigate the remote possibility that a combination of benzaldehyde and benzyl alcohol prevented benzyl alcohol dehydrogenase synthesis. However, benzyl alcohol dehydrogenase was induced with similar kinetics by 1.5 mM-benzyl alcohol alone or by 1.5 mM-benzyl alcohol plus 0.05 or 0.5 mM-benzaldehyde, indicating that benzaldehyde did not cause the repression of benzyl alcohol dehydrogenase synthesis observed during phenylglyoxylate metabolism.

Synthesis of benzyl alcohol dehydrogenase in *A. calcoaceticus* was repressed by 27 to 34% in the presence of 1.5 mM-benzoate (Table 2), and no endogenous benzyl alcohol dehydrogenase activity was detected. With mutant NA21, benzyl alcohol dehydrogenase synthesis was not at all repressed in the presence of benzoate. However, as with the wild-type strain, endogenous benzyl alcohol dehydrogenase synthesis was not observed in the presence of benzoate.

The presence of succinate caused severe transient repression of benzyl alcohol dehydrogenase synthesis in the wild type (Table 2) and in mutant strains NA36 and NA21. No benzyl alcohol dehydrogenase activity (even endogenous levels) was detected during the initial 40 min growth, but then induction commenced at unrepressed rates.

Thiophenoxyacetate, a gratuitous inducer of the R1 enzymes (Livingstone & Fewson, 1972), caused 50% repression of benzyl alcohol dehydrogenase synthesis in the wild-type strain (Table 2) but no repression in strain NA36.

Pre-induced inocula. Pre-induction of the inoculum with phenylglyoxylate resulted in total repression of benzyl alcohol dehydrogenase in the presence or absence of phenylglyoxylate (Fig. 6a). This repression was also observed when 0.5 mM-benzaldehyde was used as inducer.

Benzyl alcohol dehydrogenase synthesis in *A. calcoaceticus* pre-induced by benzoate was repressed by 65 to 75% in the presence of benzoate during the initial 80 min growth and then repression was relieved (Fig. 6b). The differential rate of synthesis of benzyl alcohol dehydrogenase in the absence of benzoate was initially the same as for an uninduced inoculum, but after 20 min growth the rate of enzyme synthesis decreased transiently to 26 to 35% of the rate with an uninduced inoculum. In contrast, an inoculum of strain NA21 prepared by growth in the presence of benzoate subsequently synthesized benzyl alcohol dehydrogenase at unrepressed rates in the presence or absence of benzoate. As with the wild-type organism, no endogenous enzyme activity was detected in the culture containing benzoate.

Benzyl alcohol dehydrogenase was repressed in *A. calcoaceticus* gratuitously pre-induced by thiophenoxyacetate (Fig. 6c). The extent of repression was approximately proportional to the extent of pre-induction. Mutant NF1408 was also repressed after pre-induction by thiophenoxyacetate, but mutant NA36 was not.

The repression during growth of phenylglyoxylate- or thiophenoxyacetate-induced bacteria (Fig. 6a, c) in the absence of added phenylglyoxylate suggested that phenylglyoxylate did not itself cause the repression, unless perhaps a very small amount carried through the harvesting and washing procedures might be sufficient to cause the effect. To test this, a culture growing on L-glutamate (a) was divided into two and phenylglyoxylate (final concn 1.5 mM) was added to one half of the culture (b); after about 5 min both cultures were harvested and washed. Chloramphenicol (final concn 140 μ M) was added to a second L-glutamate culture (c) which was divided into two, and phenylglyoxylate was added to one

Fig. 5

Fig. 6

Fig. 5. Effect of phenylglyoxylate on benzyl alcohol dehydrogenase induction in (a) mutant NA36 and (b) mutant NA21. Inocula were prepared by growth on L-glutamate and added to 5 mM-L-glutamate/salts media (500 ml) containing 1.5 mM-benzyl alcohol (○) or 1.5 mM-benzyl alcohol plus 1.5 mM-phenylglyoxylate (△). Turbidities and benzyl alcohol dehydrogenase activities were measured at intervals. Values quoted on the graphs are differential rates of synthesis [munits (mg protein)⁻¹] corrected for endogenous synthesis of enzyme.

Fig. 6. Effect of pre-induction by phenylglyoxylate, benzoate or thiophenoxyacetate on the subsequent induction of benzyl alcohol dehydrogenase in *A. calcoaceticus* NCIB8250.

(a) A glutamate culture was induced by adding phenylglyoxylate (final concn 1.5 mM) 90 min before harvesting, and was used as inoculum for 5 mM-L-glutamate/salts medium (500 ml) containing no addition (●), 1.5 mM-benzyl alcohol (○), 1.5 mM-phenylglyoxylate (△), or 1.5 mM-benzyl alcohol plus 1.5 mM-phenylglyoxylate (□). Endogenous benzyl alcohol dehydrogenase activity (●) has not been subtracted.

(b) A glutamate culture was induced by adding benzoate (final concn 1.5 mM) 120 min before harvesting and was used as inoculum for 5 mM-L-glutamate/salts medium (500 ml) containing 1.5 mM-benzyl alcohol (○), 1.5 mM-benzoate (△) or 1.5 mM-benzyl alcohol plus 1.5 mM-benzoate (□). The results for the culture without benzoate were corrected by subtraction of control values for enzyme activity in glutamate/salts medium (i.e. endogenous synthesis).

(c) Inocula were pre-induced for R1 enzymes to different extents by adding thiophenoxyacetate (final concn 1.5 mM) to the L-glutamate culture 40 min (○), 80 min (△) or 130 min (□) before harvesting. These inocula were added to 5 mM-L-glutamate/salts medium (500 ml) containing 1.5 mM-benzyl alcohol.

Values quoted on the graphs are differential rates of enzyme synthesis [munits (mg protein)⁻¹].

half (d). Cultures c and d were incubated under growth conditions for a further 30 min and then harvested and washed as usual. The four inocula were then grown in salts medium containing 5 mM-L-glutamate plus 1.5 mM-benzyl alcohol and samples were assayed for benzyl alcohol dehydrogenase and L(+)-mandelate dehydrogenase activities. The differential rate of synthesis of benzyl alcohol dehydrogenase was similar in all four cultures. The L(+)-mandelate dehydrogenase activity in culture b was less than 10 munits (mg protein)⁻¹.

These results confirm that phenylglyoxylate carried over in the inoculum was not sufficient to cause the repression observed in Fig. 6(a).

Effect of cyclic AMP. Addition of 10 mM-cyclic AMP to cultures growing in medium containing 5 mM-L-glutamate plus 1.5 mM-benzyl alcohol depressed the differential rate of synthesis of benzyl alcohol dehydrogenase by about 50%. Preliminary experiments showed that concentrations of cyclic AMP up to 25 mM had the same effect. Cyclic AMP (10 mM) did not overcome the repression caused by phenylglyoxylate, benzoate or succinate. The nucleotide probably entered the bacteria because it slightly increased the rate of growth on a number of compounds and had an effect on respiration (L. Grieve, C. A. Hills & C. A. Fewson, unpublished observations).

Repression in strains constitutive for the R₁ enzymes. Moyes & Fewson (1976) isolated constitutive mutants by selecting on the basis of ability to grow on phenylglyoxylate in the presence of 2-phenylpropionate, which is an anti-inducer of the regulon (Fewson & Foote, 1976). Strain HMM5 is magno-constitutive for the R₁ enzymes and could not grow on benzyl alcohol or form benzyl alcohol dehydrogenase in the presence of benzyl alcohol (Table 3). In other words, although it had been isolated solely on the basis of constitutivity of the R₁ enzymes, it was fully repressed for benzyl alcohol dehydrogenase. This mutant grew slowly on benzaldehyde, presumably oxidizing it with benzaldehyde dehydrogenase I.

When mutant HMM5 was shaken in benzyl alcohol/salts medium, growth occurred after 1 to 4 days; this was apparently primarily due to outgrowth of second-stage mutants. Mutant J8, a typical example of this class of second-stage mutants, was selected on the basis of its ability to grow on benzyl alcohol/salts agar but it could not grow on L(+)-mandelate or on phenylglyoxylate (Table 3). It was inducible for benzyl alcohol dehydrogenase, which explains why it could grow on benzyl alcohol. Table 3 shows that mutant J8 had not reverted to inducibility of the R₁ enzymes, but rather it could not form L(+)-mandelate dehydrogenase or phenylglyoxylate carboxy-lyase either constitutively or inducibly. The mutant constitutively formed very high amounts of benzaldehyde dehydrogenase I. This suggests that regulon R₁ has some sort of internal division which allows non-coordinate control under some circumstances.

Third-stage mutants could be obtained by shaking mutant J8 in phenylglyoxylate/salts medium. One of these mutants, J8/R₁ (Table 3), was selected on phenylglyoxylate/salts agar; it was once again constitutive for all three R₁ enzymes, and was repressed for benzyl alcohol dehydrogenase and unable to grow on benzyl alcohol.

Involvement of the R₁ system in the repression of benzyl alcohol dehydrogenase is confirmed by the isolation of these successive mutants. In each case, selection for constitutivity or for ability to grow on phenylglyoxylate or benzyl alcohol, led (quite independently) to reciprocal expression of benzyl alcohol dehydrogenase and of phenylglyoxylate carboxy-lyase and L(+)-mandelate dehydrogenase.

Mutants meso-constitutive for the R₁ enzymes were only partially repressed for benzyl alcohol dehydrogenase (Fig. 7). In the one case examined (mutant 26c) there seemed to be some release from repression, so that the degree of repression decreased with time in the presence of benzyl alcohol. This was similar to the pattern with bacteria pre-induced with thiophenoxyacetate (Fig. 6c).

Constitutive mutants were derived from mutant NF1408 by growth in the presence of 2-phenylpropionate. These lacked L(+)-mandelate dehydrogenase, but were constitutive for phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I. These strains were also repressed for benzyl alcohol dehydrogenase (Fig. 7) and were unable to grow on benzyl alcohol except after a long lag.

Correlation between expression of phenylglyoxylate carboxy-lyase and benzyl alcohol dehydrogenase. The results described so far pointed to a direct involvement of phenylglyoxylate carboxy-lyase (or of something synthesized co-ordinately with it, but not one of the other R₁ enzymes) in repression of benzyl alcohol dehydrogenase. An attempt was made to

Fig. 7. Correlation between phenylglyoxylate carboxy-lyase and benzyl alcohol dehydrogenase activities in the wild type and mutant strains of *A. calcoaceticus*. Bacteria were grown for 2 h in salts medium containing 5 mM-L-glutamate plus 2 mM-benzyl alcohol with or without additional compounds. All the results were obtained in a single experiment. ○, Wild type pre-induced with thiophenoxyacetate for 30 to 150 min (five inocula) and inoculated into the standard medium; ●, wild type pre-induced with phenylglyoxylate for 30 min and inoculated into the standard medium; ●, uninduced wild type inoculated into medium containing 5 mM-phenylglyoxylate; ○, uninduced wild type inoculated into medium containing 2 mM-phenylglyoxylate plus 2 mM-2-phenylpropionate; ●, uninduced wild type inoculated into the standard medium. Nine mutants of the wild type (△) and twelve mutants of strain NF1408 (□) selected for the ability to grow on phenylglyoxylate in the presence of 2-phenylpropionate, but not screened for constitutivity of the R1 enzymes, were inoculated (without pre-induction) into the standard medium. The replicate points ● and ● with lower specific activities were obtained from flasks in which larger inocula were used.

see if there was any quantitative correlation. The wild type and several mutants were grown under conditions giving different degrees of expression of the two regulons (Fig. 7). In general there was an inverse relationship between the specific activities of benzyl alcohol dehydrogenase and phenylglyoxylate carboxy-lyase. Mutants able to grow in the presence of 2-phenylpropionate (△, □), for instance, were repressed in approximate proportion to their constitutivity; those which were not constitutive (and presumably had some other mechanism for growing in the presence of 2-phenylpropionate) were induced for benzyl alcohol dehydrogenase. Some of the mutants grew at different rates from the wild type and some of the additions caused altered growth rates, so measurements of differential rates of synthesis might have been more accurate than determination of specific activities at a single time, but this was not possible for so many samples.

Repression is not caused by inhibition of uptake or of enzyme activity. A culture growing on L-glutamate was induced with 1.5 mM-benzyl alcohol for 60 min, harvested and washed, and then grown for a further 60 min in L-glutamate plus 1.5 mM-phenylglyoxylate. The bacteria were harvested and resuspended in salts medium containing 1.5 mM-benzyl alcohol plus 140 µM-chloramphenicol. Measurement of benzyl alcohol utilization by methods similar to those described by Cook *et al.* (1975) showed that the growth in the presence of phenylglyoxylate had not impaired the ability to metabolize benzyl alcohol [170 nmol min⁻¹ (mg protein)⁻¹]. Similarly, bacteria pre-induced in the presence of benzyl alcohol and then grown in salts medium containing 5 mM-L-glutamate plus 1.5 mM-phenylglyoxylate plus 1.5 mM-benzyl alcohol, continued to utilize benzyl alcohol at a constant rate (Fig. 8*b*) although induced synthesis of new benzyl alcohol dehydrogenase was prevented (Fig. 8*a*).

An experiment was also done to check that measurement of benzyl alcohol dehydrogenase was not interfered with by some component of bacteria induced for the R1 enzymes: this sort

Fig. 8. Effect of phenylglyoxylate on (a) benzyl alcohol dehydrogenase synthesis and (b) benzyl alcohol utilization by a culture pre-induced by benzyl alcohol. *Acinetobacter calcoaceticus* NCIB8250 was grown in 5 mM-L-glutamate medium and induced by the addition of benzyl alcohol (final concn 1.5 mM) 60 min before harvesting. This was used as inoculum for 5 mM-L-glutamate/salts media (500 ml) containing 1.5 mM-benzyl alcohol (○) or 1.5 mM-benzyl alcohol plus 1.5 mM-phenylglyoxylate (△). A control culture, showing synthesis of benzyl alcohol dehydrogenase in the absence of added benzyl alcohol was not included in this experiment and, therefore, these results have not been corrected for endogenous benzyl alcohol dehydrogenase synthesis. Figure 3 shows the pattern of endogenous benzyl alcohol dehydrogenase synthesis in a culture pre-induced to a similar extent. Values quoted on the graphs are differential rates of enzyme synthesis [munits (mg protein)⁻¹].

Table 4. *Effect of rifampicin and phenylglyoxylate on the synthesis of benzyl alcohol dehydrogenase*

A non-induced inoculum was added to flasks containing 5 mM-L-glutamate/salts medium (200 ml). After 20 min growth, benzyl alcohol (final concn 1.5 mM) with or without phenylglyoxylate (final concn 1.5 mM) was added to each culture and they were grown for a further 20 min. Rifampicin and phenylglyoxylate were then added (final concn 7.3 μ M and 1.5 mM, respectively) and samples were taken for enzyme assay after a further 20 min stirring (i.e. 60 min after inoculation).

Addition at 20 min	Addition at 40 min	Benzyl alcohol dehydrogenase activity after 60 min [munits (mg protein) ⁻¹]
Benzyl alcohol	Rifampicin	113
Benzyl alcohol	Rifampicin + phenylglyoxylate	105
Benzyl alcohol + phenylglyoxylate	Rifampicin	12

of effect would lead to apparent repression. Bacteria grown on L-glutamate, L(+)-mandelate, or benzyl alcohol were suspended in 0.08 M-Na₄P₂O₇ buffer (pH 8.5) plus 2 mM-dithiothreitol and ultrasonically disrupted. The extracts were assayed for benzyl alcohol dehydrogenase (Livingstone *et al.*, 1972) and samples of different extracts were mixed 2 and 40 min before assay. In no case was there any evidence for interference with the measurement of benzyl alcohol dehydrogenase activity.

Effects of rifampicin and chloramphenicol. Phenylglyoxylate had to be present during transcription in order to cause repression of benzyl alcohol dehydrogenase (Table 4).

Further attempts to use chloramphenicol to separate effects on transcription and translation were prevented by the observation that pre-treatment of bacteria with chloramphenicol (15 μ M) caused a subsequent rapid synthesis of 'endogenous' benzyl alcohol dehydrogenase in the absence of any inducer. It is possible that this was due to the degradation of a repressor protein during the harvesting and washing procedure (which may be responsible for the usual small burst of synthesis of 'endogenous' enzyme; e.g. Fig. 3) and the prevention by chloramphenicol of its re-synthesis. Chloramphenicol (15 μ M) prevented appearance of benzyl alcohol dehydrogenase activity after addition of 1.5 mM-benzyl alcohol to bacteria growing in glutamate/salts medium, so enzyme induction must involve *de novo* protein synthesis.

DISCUSSION

There appear to be at least two distinct types of repression of benzyl alcohol dehydrogenase in *Acinetobacter calcoaceticus*. The first is associated with metabolism of L(+)-mandelate to benzoate; this is illustrated by repression by phenylglyoxylate in mutant NA21 which cannot further metabolize benzoate (Table 2a; Fig. 5b). The second type of repression occurs below the level of benzoate.

The repression of benzyl alcohol dehydrogenase by a component of the R₁ system is consistent with the patterns of growth and metabolism in batch culture (Cook *et al.*, 1975; Beggs *et al.*, 1976). The following are ruled out as necessary or sufficient agents of the repression: (i) L(+)-mandelate [there was no repression in the presence of L(+)-mandelate in mutants lacking L(+)-mandelate dehydrogenase (Table 2a)]; (ii) phenylglyoxylate [(a) there was no repression in the presence of phenylglyoxylate in mutants lacking phenylglyoxylate carboxy-lyase (Table 2a); (b) addition of 2-phenylpropionate prevented repression with phenylglyoxylate in the wild type (Fig. 7)]; (iii) benzaldehyde [addition of benzaldehyde did not prevent induction of benzyl alcohol dehydrogenase by benzyl alcohol]; (iv) L(+)-mandelate dehydrogenase [(a) there was no repression by phenylglyoxylate in blocked mutants NF1408 and NF0410 (Table 2a); (b) there was no repression in the presence of phenylglyoxylate in NF1706 and NA36 or in pre-induced NA36 although they contain the enzyme (Table 2a); (c) repression occurred in constitutive mutants derived from NF1408 which had no L(+)-mandelate dehydrogenase (Fig. 7)]; (v) benzaldehyde dehydrogenase I [(a) there was no repression in mutant J8 which was magno-constitutive for this enzyme (Table 3); (b) there was no repression in mutants NF1706 and NA36 in the presence of phenylglyoxylate or in pre-induced NA36, although this enzyme would be present (Table 2a)]; (vi) benzoate [(a) there was no repression by benzoate in mutant NA21; (b) even in the wild type, benzoate caused relatively little repression (Table 2b)].

Experiments with pre-induced bacteria (Fig. 6) and constitutive mutants also made it unlikely that the repression was caused by an intermediate of the pathway. The results in this paper, which confirm and extend previous results (Beggs *et al.*, 1976), suggest that there was no inhibition of benzyl alcohol dehydrogenase activity or of uptake of benzyl alcohol (e.g. Fig. 8). Thus all the likely candidates except phenylglyoxylate carboxy-lyase were eliminated, and this enzyme was indirectly implicated by the observation that no repression by phenylglyoxylate occurred in blocked mutants NF1706, NA36, NF2405 or NF0113 (whose common feature is that they lack the carboxy-lyase) nor was there repression in pre-induced mutant NA36. More positive evidence is that there was a correlation between the activity of phenylglyoxylate carboxy-lyase and the extent of repression for a range of mutants and several different conditions of induction (Fig. 7, Table 3 etc.).

There are several examples of enzymes controlling their own synthesis or the synthesis of other enzymes (e.g. Magasanik *et al.*, 1974; Goldberger, 1974) but it is difficult to see any selective advantage in repression of benzyl alcohol dehydrogenase by phenylglyoxylate carboxy-lyase in *A. calcoaceticus* since it leads to a decreased growth rate (Beggs *et al.*, 1976). There are various possible explanations: (i) the substrates for the enzymes in Nature

may be substituted mandelates or benzyl alcohols, or some rather different compounds, for which it would be an advantage for R₁ to dominate R₂; (ii) the control may arise as an accidental consequence of some other features of regulation; (iii) it may be an evolutionary relic arising, for instance, because of the ways in which the enzymes came to have their present functions, possibly by recruitment from other pathways (e.g. Jensen, 1976). [Unambiguous proof that the repression is caused by the carboxy-lyase will demand a more direct attack; at the moment we cannot rule out the possibility that repression is caused by a compound synthesized co-ordinately with phenylglyoxylate carboxy-lyase, or is a consequence of some related event.

Repression at high growth rates (Fig. 2) or during metabolism of compounds such as benzoate, catechol, succinate or acetate (Table 2*b*) may all involve the same mechanism, which appears to be an example of catabolite repression. Self-repression during benzyl alcohol metabolism was demonstrated by the high specific activity in mutant NA21 which could not further metabolize benzoate formed from benzyl alcohol (Table 2*a*) and by the intermediate period of repression after pre-induction by benzoate (Fig. 6*b*), presumably caused by a rapid flux of metabolites once sufficient R₂ enzymes had been formed.

This paper describes the regulation of benzyl alcohol dehydrogenase. We presume that benzaldehyde dehydrogenase II shows parallel regulation since in all our early experiments these two enzymes were regulated co-ordinately (Livingstone *et al.*, 1972).

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REFERENCES

- BAKER, J. (1968). Low cost continuous culture apparatus. *Laboratory Practice* **17**, 817-824.
- BEGGS, J. D. & FEWSON, C. A. (1974). Repression of the enzymes converting benzyl alcohol into benzoate in *Acinetobacter calcoaceticus* N.C.I.B. 8250. *Biochemical Society Transactions* **2**, 924-925.
- BEGGS, J. D., COOK, A. M. & FEWSON, C. A. (1976). Regulation of growth of *Acinetobacter calcoaceticus* NCIB8250 on benzyl alcohol in batch culture. *Journal of General Microbiology* **96**, 365-374.
- CLARKE, P. H., HOULDSWORTH, M. A. & LILLY, M. D. (1968). Catabolite repression and the induction of amidase synthesis by *Pseudomonas aeruginosa* 8602 in continuous culture. *Journal of General Microbiology* **51**, 225-234.
- COOK, A. M., BEGGS, J. D. & FEWSON, C. A. (1975). Regulation of growth of *Acinetobacter calcoaceticus* NCIB8250 on L-mandelate in batch culture. *Journal of General Microbiology* **91**, 325-337.
- DEAN, A. C. R. (1972). Influence of environment on the control of enzyme synthesis. *Journal of Applied Chemistry and Biotechnology* **22**, 245-259.
- FEWSON, C. A. (1967). The growth and metabolic versatility of the Gram-negative bacterium NCIB-8250 ('*Vibrio* 01'). *Journal of General Microbiology* **46**, 255-266.
- FEWSON, C. A. & FOOTE, A. (1976). Inhibition of induction of the mandelate regulon in *Acinetobacter calcoaceticus* NCIB8250. *Proceedings of the Society for General Microbiology* **3**, 177.
- GOLDBERGER, R. F. (1974). Autogenous regulation of gene expression. *Science* **183**, 810-816.
- HARVEY, N. L., FEWSON, C. A. & HOLMS, W. H. (1968). Apparatus for batch culture of micro-organisms. *Laboratory Practice* **17**, 1134-1136.
- JENSEN, R. A. (1976). Enzyme recruitment in evolution of new function. *Annual Review of Microbiology* **30**, 409-425.
- KENNEDY, S. I. T. & FEWSON, C. A. (1968*a*). Enzymes of the mandelate pathway in bacterium N.C.I.B. 8250. *Biochemical Journal* **107**, 497-506.
- KENNEDY, S. I. T. & FEWSON, C. A. (1968*b*). Metabolism of mandelate and related compounds by bacterium NCIB8250. *Journal of General Microbiology* **53**, 259-273.
- LIVINGSTONE, A. (1970). *Induction and activity of the mandelate pathway enzymes in bacterium NCIB8250*. Ph.D. thesis, University of Glasgow.
- LIVINGSTONE, A. & FEWSON, C. A. (1972). Regulation of the enzymes converting L-mandelate into benzoate in bacterium N.C.I.B. 8250. *Biochemical Journal* **130**, 937-946.
- LIVINGSTONE, A., FEWSON, C. A., KENNEDY, S. I. T. & ZATMAN, L. J. (1972). Two benzaldehyde dehydrogenases in bacterium N.C.I.B. 8250. Distinguishing properties and regulation. *Biochemical Journal* **130**, 927-935.
- MAGASANIK, B., PRIVAL, M. J., BRENCHELEY, J. E., TYLER, B. M., DELEO, A. B., STREICHER, S. L., BENDER, R. A. & PARIS, C. G. (1974). Glutamine synthetase as a regulator of enzyme synthesis. *Current Topics in Cellular Regulation* **8**, 119-138.
- MOYES, H. M. & FEWSON, C. A. (1976). Constitutivity of the mandelate enzymes in *Acinetobacter calcoaceticus* N.C.I.B. 8250 and its effect on the synthesis of benzyl alcohol dehydrogenase. *Biochemical Society Transactions* **4**, 1105-1106.
- REINER, A. M. (1971). Metabolism of benzoic acid by bacteria: 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid is an intermediate in the formation of catechol. *Journal of Bacteriology* **108**, 89-94.