Effect of Growth Conditions on Phenazine Production by *Pseudomonas phenazinium*

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Phenazine production by *Pseudomonas phenazinium* was not reduced by high concentrations of phosphate during batch culture (cf. *Pseudomonas aeruginosa*) but was inhibited by high concentrations of $(\text{NH}_4)_2\text{SO}_4$ (1 to 10 g l⁻¹). In carbon-, sulphate-, magnesium- and potassium-depleted medium phenazine production paralleled growth. In continuous culture, phenazine production occurred over a wide range of growth rates under various nutrient-limiting conditions. At low growth rates cell resources were conserved and cell mass was maintained at the expense of secondary metabolite production. Low oxygen tensions and the addition of phenylalanine (0.1 g l⁻¹) each inhibited phenazine production.

INTRODUCTION

Ten different phenazines are produced by *Pseudomonas phenazinium* wild-type. The major one is the purple pigment, iodinin, whereas 9 other phenazines are only produced in trace amounts. Evidence has been obtained for a branched biosynthetic pathway (Byng & Turner, 1976, 1977) (Fig. 1). Mutants with altered patterns of phenazine production are available (Byng & Turner, 1976). Phenazines are secondary products of the shikimate pathway, chorismate being the branchpoint compound (Calhoun *et al.*, 1972; Longley *et al.*, 1972).

Although phenazines are produced in copious quantities by *P. phenazinium* under certain conditions, e.g. growth on a minimal salts medium containing L-threonine (Bell & Turner, 1973; Byng & Turner, 1975), their significance is uncertain.

Several reports have described the effects of growth conditions on phenazine production by pseudomonads, e.g. medium composition in the case of *Pseudomonas aureofaciens* (Korth, 1973, 1974) and temperature and oxygen saturation in the case of *Pseudomonas aeruginosa* (Kanner *et al.*, 1978). Low concentrations of phosphate in the growth medium for *P. aeruginosa* stimulated pyocyanin production (Ingledew & Campbell, 1969). The advantages to the producing organism of phenazine production have never been assessed.

In this paper we describe the effects of growth conditions on phenazine production by *P. phenazinium* in batch and continuous culture.

METHODS

Micro-organisms. *Pseudomonas phenazinium* (NCIB 11027) was obtained from the National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, U.K. It was originally isolated in this laboratory from a soil sample (Bell & Turner, 1973). Several mutants with altered patterns of phenazine production have been isolated (Byng & Turner, 1976). Mutant D34 was chosen for growth in continuous culture as it produced soluble phenazines from both branches of the phenazine pathway (Fig. 1). The major phenazine produced by the wild-type pseudomonad is water-insoluble iodinin which aggregates, adheres to surfaces and precludes homogeneous sampling during growth.

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Fig. 1. Scheme for phenazine biosynthesis in *P. phenazinum* (from Byng & Turner, 1977). The probable positions of lesions in mutant D34 are indicated. This strain produces no phenazine *N*-oxides and it is possible that the same enzyme is responsible for oxidation of the 1,6- and 1,8-dihydroxyphenazines (Byng & Turner, 1976). Thus both lesions in the pathway may be produced by a single mutation. However, further evidence is needed to clarify this.

Media and culture conditions. For phenazine production *P. phenazinum* strains were grown in liquid culture on basal carbon-limiting medium containing 2 g L-threonine, 1.2 g Na_2SO_4 , 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 8.5 g KH_2PO_4 made up to 1 litre with glass-distilled water and adjusted to pH 5.5 with concentrated NaOH. All other media were modifications of this. Phosphate-depleted medium contained 7.5 g morpholinoethane sulphonate buffer and 0.25 g $\text{KH}_2\text{PO}_4 \text{ l}^{-1}$; nitrogen-depleted medium contained 2.5 g glycerol and 0.5 g threonine l^{-1} ; sulphate-depleted medium contained 0.1 g $\text{MgCl} \text{ l}^{-1}$ to replace MgSO_4 ; magnesium-depleted medium contained 0.1 g $\text{Na}_2\text{SO}_4 \text{ l}^{-1}$ to replace MgSO_4 and potassium-depleted medium contained 8.5 g $\text{NaH}_2\text{PO}_4 \text{ l}^{-1}$ to replace KH_2PO_4 .

Liquid cultures (100 ml) were grown in 250 ml conical flasks and shaken at 30 °C on a gyratory shaker. Growth of cultures was measured spectrophotometrically at 600 nm. *Pseudomonas phenazinum* mutant D34 was grown in continuous culture in a 2.5 l chemostat. The steady state was usually reached when 4 to 5 culture volumes of medium had passed through the vessel and was monitored by measuring the absorbance of culture samples. At each steady state a 1 ml sample was taken for viable cell counts, a 20 ml sample for phenazine estimation and a 50 ml sample for cell mass estimation. Chemostat temperature was maintained at 30 °C by a temperature probe and pH was not controlled during growth. Oxygen saturation was measured by an oxygen probe. Complete saturation was achieved by bubbling compressed air through the sterile medium at a rate of 4.5 l min^{-1} until the oxygen level remained constant. Oxygen saturation is expressed as a percentage of this value.

Measurement of cell dry mass. A modification of the method of Hadjipetrou *et al.* (1964) was used. Samples (50 ml) were centrifuged at 12000 *g* for 20 min at 0 °C, the pellet was washed with distilled water and recentrifuged. The pellet was then dried to constant weight at 90 °C in pre-weighed vials.

Extraction of phenazines. Phenazines were extracted from acidified cultures (pH 2) 24 h after growth had ceased by adding an equal volume of chloroform and shaking on a gyratory shaker for at least 6 h. The chloroform layer

was then separated from the aqueous layer using Whatman phase-separating paper and the extraction repeated.

Estimation of phenazines. Because of the difficulty in purifying enough of each phenazine to measure its molar absorbance coefficients it was only possible to estimate total phenazine production. Therefore phenazines were estimated spectrophotometrically in chloroform solution. Phenazine 1,6-dicarboxylate and phenazine 1-carboxylate were estimated by measuring absorbance at 254 nm and 250 nm, respectively. The absorption maximum of 1,6-dihydroxyphenazine occurred at 273 nm and that of iodinin at 290 nm. 1,6-Dihydroxyphenazine *N*-oxide was estimated by measuring its absorbance at 283 nm.

By separating phenazines on silica TLC plates run in chloroform/acetic acid (9:1, v/v) (Byng & Turner, 1975) and scanning the plates at the above wavelengths it was shown that each of these wavelengths was characteristic of one phenazine fraction, and that there was no interference by other products absorbing at these wavelengths.

RESULTS

Effect of medium composition on phenazine production

Over a range of carbon (0 to 15 g threonine l⁻¹), sulphate (0 to 10 mg MgSO₄ l⁻¹), magnesium (0 to 1 mg MgSO₄ l⁻¹), potassium (0 to 25 mg KH₂PO₄ l⁻¹) and phosphate (0 to 5 mg KH₂PO₄ l⁻¹) concentrations, phenazine production in wild-type *P. phenazinium* paralleled growth, i.e. a depletion of none of the components tested caused increased production. Under conditions of nitrogen and sulphate depletion, however, phenazine 1-carboxylate (normally present in only trace amounts) was the major phenazine formed rather than iodinin.

When carbon-depleted medium containing 0.5 g threonine l⁻¹ was supplemented with glycerol (0 to 10 g l⁻¹), to produce nitrogen-depleted conditions, maximal phenazine production was seen at about 1.0 g glycerol l⁻¹, although growth was maximal only at 2.0 g glycerol l⁻¹ (Fig. 2).

The addition of (NH₄)₂SO₄ (1 to 10 g l⁻¹) to L-threonine medium (pH 5.5) dramatically reduced iodinin production without affecting growth (Fig. 3). Over this range of (NH₄)₂SO₄ concentrations other phenazines were produced in only trace amounts.

Phenazine production in continuous culture

During carbon-limited growth of mutant D34, phenazine production occurred over a wide range of dilution rates, peaking at 0.11 h⁻¹. Cell mass remained constant over a similar range of dilution rates (Fig. 4*a*). Although cell mass was unaffected at higher rates, phenazine production dropped to virtually zero at a growth rate of 0.25 h⁻¹. Under nitrogen- and magnesium-limited conditions the decrease in phenazine production at high dilution rates corresponded to a decline in cell mass (Fig. 4*b, c*). Under all these limiting conditions the decline of phenazine production at low dilution rates corresponded to an increase in cell mass. This was especially pronounced under magnesium-limiting conditions.

Phenazine production was also maintained over a wide range of oxygen tensions on carbon-limiting medium (Fig. 5). Although it declined at saturation levels below 10% some phenazine production still occurred at the lowest oxygen saturation tested (0.4%). However, cell mass remained steady over this range of oxygen saturation.

In batch culture the addition of phenylalanine (0.1 to 0.5 g l⁻¹) to carbon-depleted medium appears to inhibit both phenazine production and growth (G. S. Byng, University of Liverpool, U.K., unpublished results). To test the possibility that decreased phenazine production was not an indirect effect of growth inhibition the phenomenon was studied in continuous culture. In carbon-limiting medium containing a low concentration of phenylalanine (0.1 g l⁻¹), phenazine production was confined to a narrower range of growth rates than in unsupplemented medium (compare Fig. 4*a* and *d*). Cell mass was comparable with that on medium lacking phenylalanine.

DISCUSSION

Nitrogen, supplied as (NH₄)₂SO₄, had an inhibitory effect on phenazine production in *P. phenazinium*. Nitrogen catabolite repression has long been known to regulate secondary metabolism in certain cases. Trihydroxytoluene production in *Aspergillus fumigatus* only occurs

Fig. 2. Effect of supplementary glycerol in carbon-depleted medium on phenazine production. Cultures of *P. phenazinium* wild-type were grown in carbon-depleted medium containing 0.5 g threonine l⁻¹ and mineral salts and various concentrations of glycerol were added so that medium became nitrogen-depleted. Maximal growth (A_{600} , ●) and phenazine production (A_{250} , □; A_{290} , ○) of these cultures were measured as described in Methods.

Fig. 3. Effect of supplementary $(\text{NH}_4)_2\text{SO}_4$ on iodinin production by *P. phenazinium*. Cultures of *P. phenazinium* wild-type were grown on threonine/mineral salts carbon-depleted medium with various amounts of $(\text{NH}_4)_2\text{SO}_4$ added. Maximal growth (A_{600} , ●) and iodinin production (A_{290} , ○) were measured as described in Methods.

when nitrogen in the medium is exhausted, and the addition of ammonium salts completely inhibits production without affecting growth or pH (Ward & Packter, 1974). The peak of phenazine production seen in glycerol-supplemented medium as the culture becomes nitrogen-depleted also suggests that the balance of carbon and nitrogen is important in regulating phenazine production.

Regulation of phenazine production in *P. phenazinium* differed from that in *P. aeruginosa* in that no inhibition by phosphate occurred. The narrowing of the range of growth rates supporting good phenazine production by phenylalanine in *P. phenazinium* also differs from *P. aeruginosa*, where tyrosine and phenylalanine enhance both phenazine production and growth (Labeyrie & Neuzil, 1981).

Phenazine production in continuous culture over a wide range of growth rates suggested that secondary metabolism is not restricted to the physiologically similar late growth phase and stationary phase in all cases. Changes in medium composition altered the optimum growth rate for phenazine production, as well as the pattern of phenazines produced. Such evidence supports

Fig. 4. Continuous culture of *P. phenazinum* mutant D34. Phenazine production (A_{250} , ●; A_{273} , ○; A_{283} , □), growth (A_{600} , △), dry cell mass (■) and log cell number (▲) were measured under various growth conditions: (a) carbon-limiting, (b) nitrogen-limiting, (c) magnesium-limiting and (d) with the addition of phenylalanine (0.1 g l⁻¹) to carbon-limiting medium.

the opinion of Demain *et al.* (1978) that secondary metabolites cannot be defined as compounds produced when growth has ceased but that the timing of secondary metabolite formation is a function of nutritional requirements. From the results it seems that when resources are limited, i.e. at low growth rates, the advantage of phenazine production is outweighed by the need to conserve cell resources and so cell mass is maintained at the expense of phenazine production. The increase in cell mass at these growth rates may be due to a shift in metabolism from phenazine production to the synthesis of intracellular metabolites, probably storage compounds. At the other extreme, the decline in phenazine production at high growth rates may reflect the fact that phenazines are unnecessary to the cell when resources are plentiful and little competition with other micro-organisms exists. At this stage this remains speculative since the function of phenazines in the producing cell remains unknown.

Fig. 5. Continuous culture of *P. phenazinium* mutant D34 under different oxygen tensions. Phenazine production (A_{250} , ●; A_{273} , ○; A_{283} , □), growth (A_{600} , △), dry cell mass (■) and log cell number (▲) were measured at steady states at a dilution rate of 0.11 h^{-1} on carbon-limiting medium.

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