Isolation of Pigmentation Mutants of *Pseudomonas phenazinium*

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SUMMARY

Pigmentation mutants of *Pseudomonas phenazinium* unable to synthesize iodinin, or producing it only in reduced amounts, were isolated. The abilities of the mutants to synthesize nine other phenazines were also altered. Cross-feeding experiments and the altered patterns of pigment production suggested metabolic relationships between the phenazine pigments, and a scheme for their biosynthesis is proposed.

INTRODUCTION

Pseudomonas phenazinium synthesizes the water-insoluble phenazine pigment iodinin, first identified as 1,6-dihydroxyphenazine 5,10-dioxide by Clemo & Daglish (1950), when grown on a variety of carbon sources, the highest yield being obtained on L-threonine (Bell & Turner, 1973). The pseudomonad also elaborates a number of relatively water-soluble pigments (Bell & Turner, 1973), the identities of which have been established (Byng & Turner, 1975). In all, 10 distinct phenazines have been recognized.

Over 30 phenazines occur naturally, all produced by bacteria, but in no case is the route of biosynthesis known. Although shikimic acid acts as a biosynthetic precursor in many such cases, for example in the synthesis of iodinin and pyocyanine, and chorismic acid is the branch-point compound leading to pyocyanine in *Pseudomonas aeruginosa* (Calhoun, Carson & Jensen, 1972; Longley *et al.*, 1972), little further information on their biosynthesis is available. A procedure for isolating pyocyanine mutants in *P. aeruginosa* has been described (Carson & Jensen, 1974), but no details of the altered patterns of pigment production by such pigment phenotypes have been described.

We report here the isolation of iodinin mutants of *P. phenazinium*. The phenazine pigments produced by a number of the mutants have been examined and cross-feeding experiments have indicated metabolic relationships.

METHODS

Bacteria. *Pseudomonas phenazinium*, isolated by Bell & Turner (1973), has been deposited with the National Collection of Industrial Bacteria, accession number NCIB11027. The mutants described are available from the authors.

Growth of bacteria. The minimal growth medium contained (g l⁻¹): KH₂PO₄, 8.43 g; Na₂SO₄ (anhydrous), 1.2 g; MgSO₄·7H₂O, 0.1 g; L-threonine, 2 g; adjusted to pH 5.5. Liquid cultures were incubated in a shaker-incubator at 30 °C. Plates containing the same medium solidified with 2 % (w/v) agar were also incubated at 30 °C.

Mutagenesis. The procedure was based on that described for mutants of a pseudomonad by Heptinstall & Quayle (1970) as modified by A. L. Herrera (unpublished). A culture of the

wild-type organism was grown on minimal medium, harvested, washed once with sterile buffer (Tris/maleate; pH 6.0; 50 mM) and resuspended in the same buffer. To 1.0 ml portions of the suspension was added an equal volume of a solution of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in Tris/maleate buffer (4 mg NTG ml⁻¹). The resulting suspension was incubated with shaking at 30 °C for 15 min. The bacteria were then harvested by centrifuging, washed twice with sterile water and resuspended in sterile minimal medium. The suspension was serially diluted and 0.1 ml samples were spread on plates to give about 100 colonies per plate. Plates were incubated for about 4 days at 30 °C before being examined for pigmentation mutants. Putative mutants, usually distinguished by the absence of iodinin from colonies, were picked off and streaked on to new plates.

Extraction and examination of phenazines. At the appropriate phase of growth the liquid cultures were adjusted to pH 2 by adding conc. HCl. An equal volume of chloroform was added to the acidified culture, and the pigments were extracted by shaking the mixture overnight at 30 °C. Usually a second extraction, with an equal volume of CHCl₃, was required to remove all traces of iodinin. The spectra of CHCl₃ extracts were examined in the u.v. and visible wavelength region. Samples of the extracts were then vigorously shaken with equal volumes of aqueous NaHCO₃ (5 %, w/v), which removes most phenazines other than iodinin, 1,6-dihydroxyphenazine and its *N*-monoxide, and the CHCl₃ phases were re-examined spectroscopically.

Samples (1.0 ml) of the original CHCl₃ extracts were chromatographed by thin-layer chromatography (t.l.c.) on silica gel G (Merck) using CHCl₃/glacial acetic acid (9:1, by vol.), and examined visually after drying. Pigment bands, provisionally identified by their *R_F* values (Byng & Turner, 1975), were eluted from the silica using appropriate solvents and examined by spectroscopic and other methods.

RESULTS

The treatment of *Pseudomonas phenazinium* with NTG resulted in about 95 % killing. The survivors were plated out for clonal isolation. Mutants with altered pigmentation were detected visually, the absence of iodinin from the morphologically compact colonies being the most obvious trait. The incidence of such mutants was 1 to 2 % among the survivors of mutagenesis.

Individual pigmentation variants were examined by growth in liquid medium. Cultures in the stationary phase were acidified and pigments were extracted and examined. About 80 % of the mutants failed to produce any phenazine in detectable amounts. Of the other mutants examined, many failed to produce iodinin in liquid or solid media, but some did produce the phenazine although in markedly reduced amounts compared with the wild type. The altered ability of mutants to synthesize iodinin was frequently compensated for by the production of other phenazines in amounts much greater than in the wild type. Mutants in which the predominant pigment was 1,6-dihydroxyphenazine, 1,6-dihydroxyphenazine 5-monoxide, 9-hydroxyphenazine 1-carboxylic acid plus 2,9-dihydroxyphenazine 1-carboxylic acid, and phenazine 1,6-dicarboxylic acid, respectively, were isolated (Table 1). The pattern of pigment production was altered in all mutants, and none of them produced only one phenazine.

Cross-feeding experiments demonstrated that iodinin can be synthesized from other phenazines. The mutant strain D34, producing predominantly but not exclusively 1,6-dihydroxyphenazine, was streaked against all of the non-pigmented strains 1Z to 26Z. In each case iodinin was formed by the non-pigmented strains at the junction of the two streaks.

Table 1. *Pigmentation mutants of Pseudomonas phenazinum*

The relative amounts of each pigment produced by the wild type are expressed as the percentage of radioactivity incorporated from L-[U-¹⁴C]threonine (150 µCi l⁻¹) following growth on the amino acid as the sole source of carbon. The amounts of the phenazines produced by mutant strains compared with the amounts produced by the wild type are expressed semiquantitatively on the basis of spectroscopic measurements. The likely position of the metabolic block in each mutant is indicated as the numbered step in the pathway proposed in Fig. 1. Numbers in parentheses indicate partial blocks.

Phenazine	Strain													
	Wild type	E7	E18	E19	B5	B8	D34	E10	E5	F11	E3	E23	F8	12 to 26Z
Phenazine 1,6-dicarboxylic acid	0.2 (+)	+	+	++	+	+	+	+	+	++	++	++	±	0
6-Hydroxyphenazine 1-carboxylic acid*	0 (0)	±	0	0	0	0	+	0	±	++	0	0	0	0
1,6-Dihydroxyphenazine	trace (±)	±	+	±	0	0	++	++	+	0	0	0	0	0
1,6-Dihydroxyphenazine 5-monoxide	trace (±)	++	+++	±	0	0	+	0	0	0	0	0	0	0
1,6-Dihydroxyphenazine 5,10-dioxide	10-15(++++)	+	±	±	±	±	0	0	0	0	0	0	0	0
Phenazine 1-carboxylic acid	1 (+)	+	+	±	++	±	+	±	±	+	±	0	++	0
9-Hydroxyphenazine 1-carboxylic acid	6 (++)	+	±	±	++	±	++	+	++	++	+	+	++	0
2,9-Dihydroxyphenazine 1-carboxylic acid	0 (0)	0	0	0	0	0	+	±	0	0	0	0	0	0
1,8-Dihydroxyphenazine*	0.3 (+)	±	0	0	+	0	0	0	0	±	0	0	+	0
Likely point of blockage (see Fig. 1)	—	(5)	(5,7)	(2,6)	(2)	(1)	(4,10)	4,10	4,9	3	2 to 5 (6)	2 to 5 (6)	2	1

* These phenazines are formed transiently or under slightly anaerobic growth conditions only (see text).

† These phenazines are not separated by the t.l.c. system employed routinely (see Methods). In the wild type, 9-hydroxyphenazine 1-carboxylic acid is the major component of the mixture.

Fig. 1. Postulated pathway for the biosynthesis of phenazine pigments by *Pseudomonas phenazinium*.

This showed that these strains were not contaminants; a variety of unrelated bacteria also capable of growth on *L*-threonine (Bell *et al.*, 1972) did not produce iodinin when plated against strain D34. The best iodinin production occurred with strains 3Z, 4Z, 8Z, 11Z, 13Z, 17Z and 18Z. The feeding of chemically pure 1,6-dihydroxyphenazine, added as a small crystal to the centre of freshly inoculated plates, resulted in iodinin formation by these seven non-pigmented strains. No iodinin was formed when these strains were tested against strains E3 and E23, neither of which excreted 1,6-dihydroxyphenazine.

Additional cross-feeding experiments between the strains listed in Table 1, together with the altered patterns of pigment production, suggested further precursor-product relationships. These are incorporated in the scheme proposed for the biosynthesis of phenazine pigments (Fig. 1). Thus, strain F11, which atypically excreted 6-hydroxyphenazine 1-carboxylic acid but no 1,6-dihydroxyphenazine, promoted iodinin formation by the non-pigmented Z strains when streaked against them. When strains E3, E23, F8 and F11, none of which produced 1,6-dihydroxyphenazine, its 5-monoxide or iodinin, were cross-fed with the 1,6-diol from strain D34, iodinin was produced by strains F8 and F11 only. It seemed likely that the metabolic block in strain F8 affected the formation of 6-hydroxyphenazine 1-carboxylic acid (step 2, Fig. 1) whereas its decarboxylation to 1,6-dihydroxyphenazine (step 3, Fig. 1) was blocked in strain F11. The formation of iodinin by F8 when streaked against F11 confirmed that 6-hydroxyphenazine 1-carboxylic acid was a precursor. The inability of E3 and E23 to form iodinin from either 6-hydroxyphenazine 1-carboxylic acid or 1,6-dihydroxyphenazine suggested that these mutants were multiply blocked (steps 2, 3, 4 and probably 5, Fig. 1). The probable positions of metabolic blocks in each mutant are indicated in Table 1.

Mutant strains E3, E19 and E23 accumulated phenazine 1,6-dicarboxylic acid but only trace amounts of some other phenazines. This suggested that the dicarboxylic acid was a common precursor, possibly a branch-point compound. The mutant F8, in which the formation of iodinin and its precursors was blocked, was found to produce relatively large amounts of phenazine 1-carboxylic acid and 9-hydroxyphenazine 1-carboxylic acid plus 2,9-dihydroxyphenazine 1-carboxylic acid (i.e. 1,8-dihydroxyphenazine 9-carboxylic acid), which suggested that these compounds were metabolically related. 1,8-Dihydroxyphenazine 10-monoxide was formed by *P. phenazinium* only after growth had ceased and appeared to be an end-product. By analogy with the formation of 1,6-dihydroxyphenazine 5-monoxide (step 4, Fig. 1), 1,8-dihydroxyphenazine was a likely precursor of its 10-monoxide, and when conditions became anaerobic towards the end of growth the 1,8-diol accumulated rather than the 10-monoxide. Mutants D34 and E10 appeared to be blocked at this point (step 10, Fig. 1), as indicated in Table 1.

The non-pigmented z strains were of interest as possible producers of the immediate precursor of the first-formed phenazine. All 26 z strains were streaked against each other. In no case did cross-feeding between non-pigmented strains result in pigment formation.

DISCUSSION

The availability of pigmentation mutants of *P. phenazinium* offers the opportunity to study the route of iodinin biosynthesis by classical procedures. Many of the strains listed in Table 1 are convenient sources of phenazines for purification and feeding to either wild type or mutant strains. The use of radioactive phenazines, obtained by administering [^{14}C]shikimate under suitable conditions, would be an extension of this approach. Podojil & Gerber (1967) have already shown that radioactive 1,6-dihydroxyphenazine and its 5-monoxide are efficient precursors of iodinin in *Brevibacterium iodinum* and Herbert, Holliman & Ibberson (1972) have similarly demonstrated the metabolism of 6-hydroxyphenazine 1-carboxylic acid to iodinin by the same bacterium. These results are consistent with the scheme proposed for the biosynthesis of phenazines (Fig. 1) on the basis of the cross-feeding experiments with pigmentation mutants.

The evidence for phenazine 1,6-dicarboxylic acid acting as a branch-point compound, and for the pathway leading to 1,8-dihydroxyphenazine 10-monoxide (Fig. 1) is incomplete but is consistent with the results. Decarboxylation, hydroxylative decarboxylation and hydroxylation reactions are well known. Even so, the involvement of phenazine 1,6-dicarboxylic acid is proposed solely on the basis of its accumulation by some mutant strains. The failure of the dicarboxylic acid to act as a precursor of iodinin, observed in the cross-feeding experiments and also reported by Herbert *et al.* (1972), may be due to permeability factors. In support of the role for phenazine 1-carboxylic acid indicated in Fig. 1, the feeding of the [^{14}C]-labelled compound to growing cultures of *P. phenazinium* resulted in the formation of radioactive 9-hydroxyphenazine 1-carboxylic acid and 2,9-dihydroxyphenazine 1-carboxylic acid. In turn, the feeding of these [^{14}C]-labelled compounds gave rise to radioactive 1,8-dihydroxyphenazine 10-monoxide (L. Swift and J. M. Turner, unpublished results).

The high incidence of non-pigmented strains suggests that the number of steps between chorismic acid and the hypothetical key intermediate common to all natural phenazines (Podojil & Gerber, 1970) may be large. It should be noted, however, that the mutant selection procedure failed to detect pigment phenotypes other than those in which the production of iodinin itself was affected. The non-pigmented strains are likely to form a heterogeneous group. Factors responsible for the initiation and termination of secondary metabolite pro-

duction are complex (Weinberg, 1970; Demain, 1973) and it is likely that the lesion in many mutants does not directly involve the route from chorismic acid. Even so it is surprising that no pigment formation occurred as a result of cross-feeding between the large number of non-pigmented strains tested. Again, impermeability of the cell membrane may be responsible or, perhaps, the lability of intermediates between chorismate and phenazines.

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