Isolation from Urine of Two *Serratia marcescens* Strains Excreting a Diffusible Yellow Pigment

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Two bacterial strains excreting a yellow pigment were isolated from human urine and identified as *Serratia marcescens*. The pigment was produced in the late exponential and early stationary phases of growth. Minimal media supplemented with tyrosine, phenylalanine, 3,4-dihydroxyphenylacetate or tryptophan, as well as complex media, induced pigment production. UV-visible spectra of the extracted pigment had peaks characteristic of 2-hydroxy-5-carboxymethylmuconate semialdehyde, produced from *meta*-cleavage of 3,4-dihydroxyphenylacetate by the enzyme 3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15). This enzyme was active when the bacteria were grown under conditions promoting pigment production. The kinetics and factors affecting pigment production are also reported.

INTRODUCTION

Some strains of the genus *Serratia* can synthesize one of the two pigments specific for that genus among enterobacteria (Grimont & Grimont, 1984; Williams & Qadri, 1980). The most common, prodigiosin, is a red, non-diffusible pigment attached to the bacterial envelope (Purkayastha & Williams, 1960; Viñas *et al.*, 1983), and the other, pyrimine, is a rose-coloured diffusible pigment present only in the biotype A4 of *S. marcescens* (Grimont & Grimont, 1984; Williams & Qadri, 1980). Many clinical isolates of *S. marcescens* are unable to produce pigments (Ball *et al.*, 1977; Clayton & von Graevenitz, 1966; Lannigan & Bryan, 1980; Ming-Jer Ding & Williams, 1983). Some authors have correlated the absence of chromogenesis with the presence of R-plasmids in *Serratia* (Holland & Dale, 1979; Platt & Sommerville, 1981). Dauenhauer *et al.* (1984) have cloned the genes involved in prodigiosin biosynthesis, and have shown that they are not exclusively contained on plasmids. We present here the characterization of two *S. marcescens* strains isolated from urine, which excrete a yellow pigment that to our knowledge has not previously been described in the family *Enterobacteriaceae*.

METHODS

Bacterial strains. *S. marcescens* ATCC 274 was used as a reference strain. Strains CY429 and CY918 were isolated from urine samples from the 'Hospital de la Santa Creu i de Sant Pau de Barcelona' and from the 'Institut Policlínic de Barcelona' respectively.

Classification and antibiotic susceptibility tests. Biochemical characterization of the strains was made following Farmer *et al.* (1985), Blazevic (1980) and Grimont & Grimont (1984). Minimal inhibitory concentrations (MICs) were measured by the Sensititre (Gibco) system.

Influence of media on pigment production. Cystine Lactose Electrolyte Deficient (CLED), MacConkey and Mueller–Hinton agars were from Difco. Trypticase Soy agar (TSA) was from BBL. Minimal medium M70 has been described elsewhere (Grimont & Grimont, 1984). GL mineral medium contained (g l⁻¹) glucose, 2; ammonium citrate, 5; K₂HPO₄, 8; MgSO₄·7H₂O, 0.5; Fe(NO₃)₃·9H₂O, 0.02; and NaCl, 0.5 (Lorén & Guinea, 1978). Bacteria were maintained on TSA slants. The influence of aromatic compounds and amino acids (all at

0.1%, w/v) on pigment production was determined in 5 ml GL, in tubes (18 × 180 mm). A 0.5 ml portion of an overnight culture in GL was used as an inoculum.

Kinetics of pigment production. Strain CY429 was used to study the kinetics of pigment production in M70 medium with glucose (0.2%, w/v) and in M70 with glucose and tyrosine (0.1%, w/v). Flasks containing 95 ml medium were inoculated with 5 ml of an overnight culture in M70 containing glucose. Incubation was carried out at 30 °C in a reciprocal shaker at 100 r.p.m. Growth was monitored as OD₅₅₀ in an Uvikon 810 (Kontron) spectrophotometer. Bacteria were removed by centrifuging and pigment production was measured in the supernatant at 380 nm.

Pigment characterization. Cultures grown in M70 containing glucose and tyrosine were harvested when A_{380} was maximal (in the late exponential phase of growth). Cultures were centrifuged at 8000 *g* for 10 min and supernatants were adjusted to pH 2 with 1 M-HCl. The pigment was extracted three times with an equal volume of ethyl acetate, and the organic layer was washed once with distilled water. The pigment was then partitioned into the aqueous layer by the addition of 1 M-NaOH. The partition procedure was repeated, and UV-visible spectra were determined under alkaline and acidic conditions (Adachi *et al.*, 1964).

Enzyme activity. For the assay of 3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15), yellow pigmented bacteria were grown in M70 containing glucose and tyrosine until the late exponential phase of growth. Strain ATCC 274 was grown on M70 containing tyrosine until the late exponential phase. Bacteria were harvested and then washed three times with 0.02 M-Tris/HCl buffer (pH 7.8) by centrifugation at 8000 *g* for 10 min. Cell pellets were resuspended in the same buffer [5 ml (g bacteria)⁻¹] and the cells were disrupted with a Labsonic 1510 (Braun) sonicator. The broken cells were centrifuged at 15000 *g* for 30 min to remove debris and the supernatant (crude extract) was used for the assay. Assays were carried out at 25 °C according to Cooper & Skinner (1980) and Sparrins *et al.* (1974). Crude extract (0.3 ml) was mixed with 2.7 ml 0.02 M-Tris/HCl buffer (pH 7.8). The reaction was started by the addition of 10 µl 10 mM-3,4-dihydroxyphenylacetate. Oxygen consumption was measured by a Clark type electrode (Rank). For routine analysis the reaction was followed by measuring the formation of 2-hydroxy-5-carboxymethylmuconate semialdehyde at 380 nm. The product of the reaction was extracted as described above and UV-visible spectra obtained from the yellow-pigmented strains and from ATCC 274 were compared with those obtained from the supernatant. All the procedures to obtain the crude extract were carried out at 4 °C. Protein was determined by the Lowry method with BSA (Sigma) as a standard.

RESULTS

Strains CY429 and CY918 were identified as *S. marcescens* (confirmation of this was kindly provided by Dr P. A. D. Grimont, Institut Pasteur, Paris, France, by using auxanograms based on 119 tests). The main biochemical differences between these strains and *S. marcescens* ATCC274 are shown in Table 1. The yellow-pigmented strains failed to grow on tyrosine, phenylalanine and 3,4-dihydroxyphenylacetate, and there were differences in their susceptibility to amikacin, tetracycline and tobramycin. Pigment was formed on MacConkey, CLED, TSA and Mueller-Hinton agars but not on solidified M70 medium containing glucose. Colour appeared around non-coloured colonies. The colour was unstable and disappeared from the media after incubation for 4 d. Only when the medium was supplemented with 3,4-dihydroxyphenylacetate, tyrosine, phenylalanine or tryptophan was pigment formed after incubation for 48 h; alanine, arginine, aspartate, cysteine, glutamine, glycine, histidine, isoleucine, leucine, methionine, proline, serine, threonine, valine, benzoate, catechol, 3,4-

Table 1. *Differential characteristics of the yellow pigmented strains*

Test	CY429	CY918	ATCC 274
Growth on:			
Tyrosine	—	—	+
Phenylalanine	—	—	+
3,4-Dihydroxy-phenylacetate	—	—	+
Tryptophan	w	w	+
NaCl (8.5%, w/v)	+	+	—
Pigment production			
Yellow	+	+	—
Prodigiosin	—	—	+

+, Growth; — no growth; w, weak growth.

Fig. 1

Fig. 1. Kinetics of yellow pigment production and growth of strain CY429. ■, Growth; ●, pigment production in M70 with glucose; ▲, pigment production in M70 with glucose and tyrosine.

Fig. 2

Fig. 2. Spectra of the extracted yellow pigment in (a) acidic and (b) alkaline conditions.

Table 2. *3,4-Dihydroxyphenylacetate 2,3-dioxygenase* activity of the strains grown in different media

Activity is expressed in nmol substrate used min⁻¹ (mg protein)⁻¹. No activity was found when any of the strains were grown on glucose.

Strain	Activity when grown on:	
	Glucose and tyrosine	Tyrosine
ATCC 274	ND	180
CY429	17	—*
CY918	16	—*

ND, Not determined.

* The yellow pigmented strains did not grow on tyrosine.

dihydroxybenzoate, 2,5-dihydroxyphenylacetate and phenol failed to promote pigment production.

Pigment was produced in the late exponential and early stationary phases of growth (Fig. 1). In liquid, as on solid media, pigment disappeared quickly. UV-visible spectra of the extracted pigment revealed characteristic peaks at 380 nm in alkaline conditions and at 325 nm in acidic conditions (Fig. 2). These spectra are identical with those of 2-hydroxy-5-carboxymethyl-muconate semialdehyde (Adachi *et al.*, 1964), which is formed by the *meta*-cleavage of 3,4-dihydroxyphenylacetate (Fig. 3) (Blackley *et al.*, 1967; Sparnins *et al.*, 1974) by the enzyme 3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15) (Ono-kamimoto, 1973; Sparnins *et al.*, 1974). Table 2 shows the activities of this enzyme in crude extracts of *S. marcescens*

Fig. 3. *meta*-cleavage of 3,4-dihydroxyphenylacetate (I) by 3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15), resulting in 2-hydroxy-5-carboxymethylmuconate semialdehyde (II).

ATCC 274 and the yellow pigmented *S. marcescens* CY429 and CY918. Extracts showed dioxygenase activity only when tyrosine was present in the medium. Strains maintained on TSA slants tended to lose the ability to produce pigment, and white strains obtained in this way were still unable to grow on tyrosine, phenylalanine and 3,4-dihydroxyphenylacetate.

DISCUSSION

Sugar fermentation tests, the ability to grow on different carbon and energy sources and other typical enterobacterial tests (Blazevic, 1980; Farmer *et al.*, 1985; Grimont & Grimont, 1984) demonstrated that strains CY429 and CY918 belong to *S. marcescens*. Growth on tyrosine as a sole carbon and energy source is a property which characterizes all strains of the genus *Serratia* studied by Grimont & Grimont (1984). The ability to grow on 3,4-dihydroxyphenylacetate as a sole carbon and energy source is a positive characteristic of *S. marcescens*. However, strains CY429 and CY918 did not grow on minimal medium with these compounds as sole carbon and energy sources. Moreover, they did not grow on phenylalanine but grew in trypticase soy broth containing 8.5% (w/v) NaCl, both of which are variable characteristics of *S. marcescens* (Grimont & Grimont, 1984).

S. marcescens CY429 and CY918 excreted a yellow diffusible pigment which to our knowledge has not been previously described in the *Enterobacteriaceae*. The two well known pigments in the genus *Serratia* are both typical secondary metabolites of this genus (Grimont & Grimont, 1984; Williams & Qadri, 1980). One of them, prodigiosin, has been extensively studied and its antibacterial and antifungal activities are well known (Torrey, 1983; Williams & Qadri, 1980). In contrast, the yellow pigment excreted by CY429 and CY918 is a metabolite of a catabolic pathway involved in the energy metabolism of aromatic compounds. Thus, this yellow pigment is produced by primary metabolism, although its formation resembles that of secondary metabolites. 2-Hydroxy-5-carboxymethylmuconate semialdehyde is formed in some bacterial species (such as *Escherichia coli*, *Pseudomonas putida*, *Acinetobacter* spp.) as an intermediate metabolite of the catabolism of aromatic compounds with a side-chain larger than a C_1 group (Blackley *et al.*, 1967; Cooper *et al.*, 1985; Cooper & Skinner, 1980; Sparnins *et al.*, 1974). The pigment is produced by the inducible enzyme 3,4-dihydroxyphenylacetate 2,3-dioxygenase which is characteristic of the homoprotocatechuate pathway for the catabolism of aromatic compounds (Blackley *et al.*, 1967; Cooper & Skinner, 1980; Sparnins *et al.*, 1974).

The results concerning pigment production, the inability to grow on tyrosine, phenylalanine and 3,4-dihydroxyphenylacetate as a sole source of carbon and energy, and the ability to grow in the presence of 8.5% (w/v) NaCl, could indicate that strains CY429 and CY918 represent a new biotype of *S. marcescens*.

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