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FATTY ACID PROFILES IN PIGMENTED AND NON-PIGMENTED STRAINS OF *S. marcescens*

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SUMMARY

Wild, pigmented strains of *Serratia marcescens* and their non-pigmented mutants were compared on the basis of fatty acid profiles and lipid content. Classic biochemical tests show only minor differences, as well as fatty acid ratio C18:C16.
The total amount of lipid synthesized and the saturated/unsaturated fatty acids ratio disclose a sharp total lipid reduction and a high percentage of unsaturated fatty acids in the pigmented strains, placing them in separated clusters compared with the nonpigmented mutants.
It is hypothesized that the synthesis of the polyacetate required for the completion of the prodigiosin molecule may result in a waste of methyl groups and thus affect the total amount of lipids.

KEY WORDS: *S. marcescens*, Fatty acids, Gas Chromatography (GC) and Fourier-Transform Infrared Spectroscopy (FT-IR)

INTRODUCTION

Serratia marcescens is a gram-negative bacterium classified within the family *Enterobacteriaceae*. The microrganism is described in detail because it is the species most commonly encountered in clinical specimens, now recognized as an opportunistic human pathogen (Davis *et al.*, 1970; Gaughran, 1969;

Wilowske *et al.*, 1970; Woodruff, 1980). Most wild-type strains produce a characteristic secondary metabolite, a diffusible red pigment (prodigiosine). Although the structure of prodigiosin, a tripyrrole, has been elucidated (Rapaport and Holden, 1962) and the biosynthetic pathway has been extensively studied (Wasserman *et al.*, 1960; Williams, 1973; Williams and Qadri, 1980), the function of prodigiosin, like that of most secondary metabolites, remains speculative (Bennet, 1983; Bennet and Ciegler, 1983; Bu'Lock, 1961; Demain, 1974; Weinberg, 1971). Comparative studies of pigmented and nonpigmented strains of *S. marcescens* reported great variations in their ability to produce the red pigment, which is formed only in the presence of oxygen and at a suitable temperature. The optimum temperature for pigment formation is not necessarily the same as that for growth. Most of the strains grow best at 30-37°C but form little or no pigment, whereas at lower temperatures, growth is poorer and pigment formation is abundant (Holmes and Aucken, 1998 ; Williams and Hearn, 1967).

The response of *Serratia* to temperature variations prevalently brings about an increase in the unsaturated and a decrease in the saturated fatty acids and sometimes in the cyclopropanes (Kates, 1964).

It has been suggested that the formation of prodigiosin might be correlated to a variation in the superficial structure (Vinas *et al.*, 1983; Parachuri and Harshey, 1987), and that the conductivity of membrane to protons is lower in non-pigmented strains than in 'wild' strains, producers of pigment (Nuria *et al.*, 1994).

Bacterial taxonomy is traditionally based on staining and some morphological parameters and on various biochemical assays that allow the differentiation of the various genera and also of the species included in the same genus.

For some years now, the use of gas chromatography of bacterial cellular fatty acids (Vannieuwenhuyze and Sandra, 1987) and, above all, of FT - IR spectroscopy has been added to these techniques (Casal *et al.*, 1979); Mantsch *et al.*, 1990; Helm *et al.*, 1991; Ismail *et al.*, 1993; Roissart and Luquet, 1994; Naumann *et al.*, 1994).

The Fourier Transform Infrared (FT - IR) by which it is possible to determine the chemical-structural characteristics of complex chemical structures, has proved to be a useful instrument for the qualitative and quantitative evaluation of the cellular components.

The aim of this paper is to evaluate whether the formation of diffusible pigment brings about a variation in the fatty acid content in prodigiosin producing strains with respect to the non-pigmented strains and whether, using the traditional biochemical methods of identification, FT-IR and GC, other differences of metabolic nature exist.

MATERIAL AND METHODS

Strains

The 5 strains of *Serratia marcescens* studied were named with the letters A - E.

Serratia marcescens isolated from the soil producer of prodigiosin (referred to as A). Mutant from strain A, unable to produce pigment, obtained by UV induction (referred to as B). *Serratia marcescens*, isolated from water, producer of prodigiosin (referred to as C). Mutant from strain C, unable to produce pigment, obtained by UV induction (referred to as D). *Serratia marcescens ATCC 8100* non-producer of pigment (referred to as E).

In order to ascertain that, on determination, all the cells would be in the same phase, the growth curve of the different strains in the culture medium used was previously evaluated. Strains B and D were cultured several times on the same medium, in order to verify the stability of the mutation that had already occurred.

Cultivation and biochemical tests

The *Serratia* strains were cultivated on nutrient agar for 18 h at a temperature of 28°C in conditions of aerobiosis. For biochemical identification all the strains were submitted to biochemical tests using the API 20 E System.

FT-IR Spectroscopy

After growth, the cells of the five strains were harvested by gentle washing of the agar surface with physiological buffered saline and washed three times in the same medium by centrifugation; the sediment obtained was lyophilized. 1.5 mg of each sample was weighed by means of a micro-analytical balance, mixed with 300 mg KBr spectroscopy IR grade. The product obtained was submitted to a pressure of 8 tons, obtaining discs

13 mm in diameter. Spectrophotometric examinations were carried out on these discs with the Perkin- Elmer FT-IR series 1700 X spectrometer by obtaining an average of 250 spectra. Normalization was obtained with abex 1.5 abs.

Gas chromatographic analysis
Lipid extraction
Amount of 0.5 g of each lyophilized sample was homogenized in 100 ml chloroform-methanol (2:1v/v) for 10 min. The mixture was filtered and 100 ml distilled water were added to the solution and then shaken in a separator funnel, the upper aqueous phase was removed by aspiration. After washing with two other aliquots of 50 ml of distilled water, the bottom phase was dried. The extract thus obtained was then weighed.
The lipid extract was brought to volume with 5 ml of chloroform. From this solution 1 ml was drawn and added to 0.1 ml internal standard (6.35 mg/ml of the methyl ester of heptanoic acid) and then dried on a waterbath.

Fatty acids
The fatty acids contained in the lipid extracts were methylated by direct transesterification using 0.5 ml of a methanol/sulphuric acid mixture (9:1).
After shaking, the mixture thus obtained was placed in an oven at 120°C for 1 h. The fatty acid methyl esters thus obtained were diluted in hexane and then analyzed, by HRGC under the following experimental conditions: gas chromatograph Fisons Mega Series 5160 equipped with a Shimadzu data processor C-R3A; fsilica column SE-54, 30 m x 0.32 mm i.d., film thickness, 0.40-0.45 μm [(Mega, Legnano (MI), Italy]; column temperature, 120°C to 150°C at 4°C/min injection mode, split; detector, FID; injector and detector temperature, 280°C; carrier gas, He 95 kPa.

RESULTS

The following observations can be drawn from the results obtained:

Biochemical tests
Table 1 shows the results obtained with the different strains assayed; the pigmented strains prove to be similar to each other and different from the mutants solely for the Voges-Proskauer test. The non-pigmented ATCC strain shows other slight differences

TABLE 1
Biochemical assays executed on five strains of Serratia marcescens *by API 20 E*

	STRAIN	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
A	*S. marcescens*	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	-	+	+	+	-
B	*S. marcescens*	+	-	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	+	+	-
C	*S. marcescens*	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	-	+	+	+	-
D	*S. marcescens*	+	-	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	+	+	-
E	*S.marcescens* ATCC 8100	+	-	+	+	-	-	-	-	-	-	-	+	+	+	+	-	+	+	-	-

(Cit, Gel and Mel). All the strains were identifiable as *Serratia marcescens*. The cluster analysis carried out with the method of amalgamation by complete linkage and distance measure by Euclidean method confirmed the homology between the wild and the mutant strains, but placed strain E, of different origin, separately (Fig. 1A). Thus, apart from the production or non-production of pigment, no noteworthy taxonomic differences between strains A, B, C and D were noted.

FT-IR Spectroscopy

Evaluation of the 1800-1000 cm^{-1} interval (Table 2), particularly in the region between 1037-1078 cm^{-1}, (- C - O -) and (- C - O - C -) groupings corresponding characteristics of carbohydrates (Wilson *et al.*, 1988), disclosed fewer polysaccharides in the wild pigment-producing strains (A, C) compared to the non-pigmented ones (B, D, E). Determinations carried out in the 3000-2830 cm^{-1} range characteristic of the lipids show a marked difference in the total lipid content, considerably higher in the three non-pigmented strains, B, D, and E. The cluster analysis underlined the existence of two distinct groups, one made up of pigmented wild strains, the other of non-pigmented strains, independently of their origin, confirming the data obtained by gas chromatography (Fig. 1B).

Gas chromatographic analysis

The fatty acid composition resulting from gas chromatography is reported in Table 3. The distribution of the single fatty acid groups (saturated, unsaturated, hydroxylated and cyclopropanic) varies in the strains also according to their origin, with a prevalence of unsaturated fatty acids in the strains isolated from water with respect to those isolated from the 'soil' (C>A), even if the total fatty acid amount is similar. When the pigmented strains were compared with the relative mutants, it was evident that, both as an absolute value and as a percentage, the diunsaturated fatty acids and the cyclopropanes were more abundant in strains A and C (respectively 2.6% and 7.3%) than in the mutants derived from them (0.9% for strain B and 1.9% for strain D). A similar prevalence can be found in the cyclopropanes which show a decrease from 5.7% to 2.4% between wild strain A and mutant B, and from 3.5% to 1% between wild strain C and mutant D.

Cluster analysis of the five classes of fatty acids resulting from gas chromatography (Fig. 1C), showed a considerable likeness between the three non-pigmented strains, D and E having more affinity. The pigmented strains A and C were instead placed in different groups. The saturated fatty acids were slightly more abundant in the non-pigmented strains than in strains A and C. As the cells of the different strains assayed were all in the same phase of growth, the differences in the fatty acid content between the pigmented and the non-pigmented strains are not attributable to the age of the culture but probably to the synthesis of the pigment.

TABLE 2

Area values, obtained by FTIR-spectroscopy, of five strains of Serratia marcescens *(see Results for more details)*

Strain	Lipids	Polysaccharides
	(3000 -2830 cm^{-1})	(1800 -1000 cm^{-1})
A	9.668	158.2
B	118.5	196.4
C	9.844	147.2
D	118.7	180.5
E	105.5	173.9

DISCUSSION

Prodigiosin derives from the condensation of an early precursor, the 4-methoxy-2-2'-bipyrrol-5 carboxyaldehyde (MBC) with a terminal ring, the 2-methyl-3-amylpyrrol (MAP) formed in the final phase of production. Whereas the precursor originates from the condensation of proline, glycine and acetate, the terminal pyrrolic ring derives from alanine and from a polyacetate whose length depends on the availability of Acetyl-CoA and influences the different kinds of synthetized prodi-

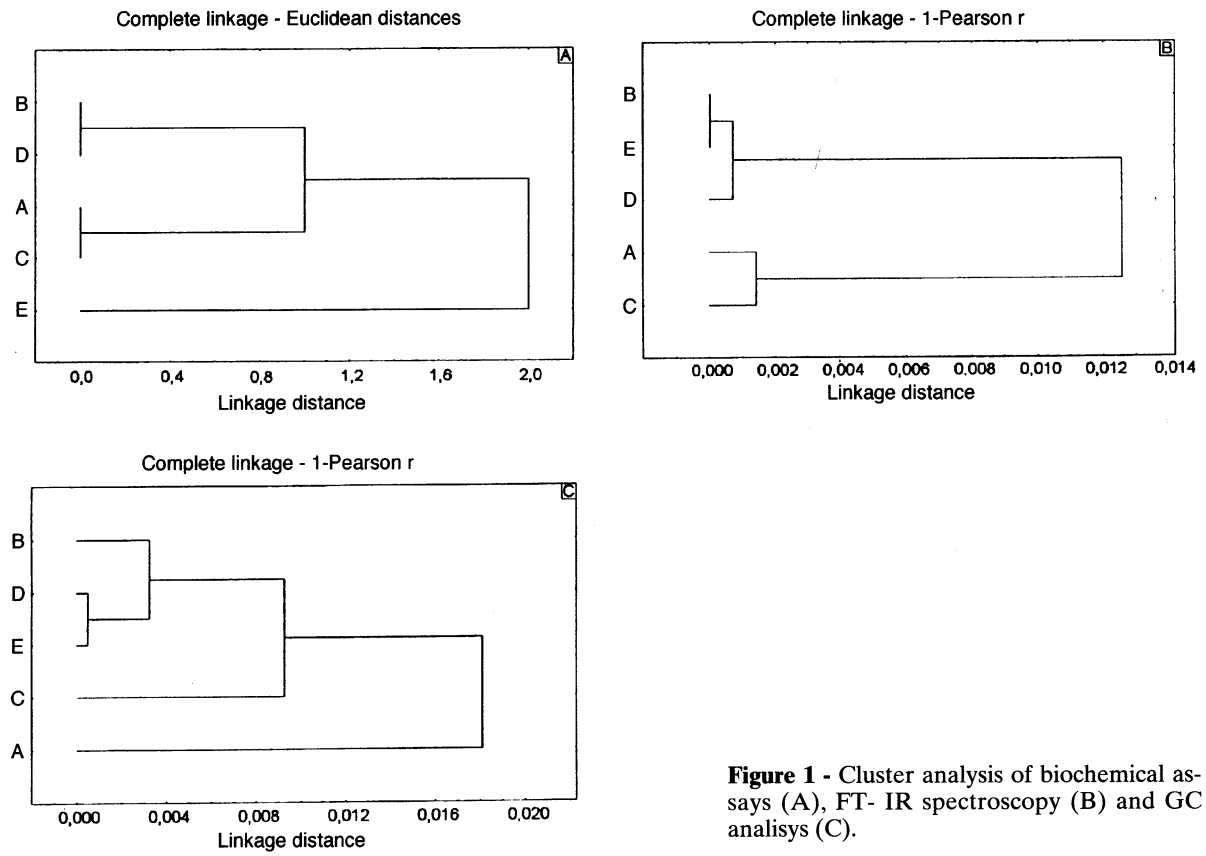

Figure 1 - Cluster analysis of biochemical assays (A), FT- IR spectroscopy (B) and GC analisys (C).

giosin (amyl, nonyl, undecil-prodigiosin) (Williams and Hearn, 1967; Cushley *et al.*, 1971; Tanaka *et al.*, 1972; Lim *et al.*, 1976).
Acetyl-CoA is necessary for the synthesis of fatty acids that proceeds with the by means of successive additions of acetylic groups to the malonyl-CoA up to the synthesis of chains with 18-20 carbon atoms.
It could be hypothesized that the synthesis of the polyacetate necessary for the completion of the prodigiosin molecule causes a considerable waste of the methyl groups and, as a consequence, a reduction of the available Acetyl-CoA, and thus of the total quantity of fatty acids in the structures of the pigmented cells.
As a matter of fact, compared to the non-pigmented mutants, the wild strains show a considerable decrease in the saturated fatty acids, together with a per cent increase in carbon atoms and of the diunsaturated acids. These factors responsible for the increase in fluidity of the membrane, might facilitate the diffusion of pigment in the culture medium and contribute to the variation in conductibility noted by Nuria *et al.* (1994) who suggest that the modification might depend upon the presence of MBC. The start of MAP synthesis might bring about a decrease in the fatty acids and an increase in membrane fluidity, with the conversion of saturated into unsaturated fatty acids and an increase in permeability. The biochemical taxonomic tests showed only slight metabolic variations between the pigmented strains and their non-pigmented mutants.
Moreover, the total ratio of C18/C16, obtained on the basis of the total content of fatty acids with 18 carbon atoms divided by that with 16 carbon atoms (Rementzis J. and Sameli, 1996), shows a close similarity between the wild

TABLE 3
Fatty acids percent composition of the five strains of Serratia marcescens

	A	B	C	D	E
1 C 10:0	0,167	0,140	-	0,102	0,120
2 C 11:0	tr	tr	-	tr	tr
3 C 2-OH 10:0	tr	tr	-	tr	tr
4 C i 12:0	tr	tr	-	tr	tr
5 C ai 12:0	tr	tr	-	tr	tr
6 C 12:1	tr	tr	-	tr	tr
7 C 12:0	2,449	2,576	tr	1,094	1,579
8 Ci 13:0		tr	-	tr	tr
9 C ai 13:0	tr	tr	-	tr	tr
10 C 13:1		tr	-	tr	tr
11 C 13:0	0,083	tr	-	tr	tr
12 C 2-OH 12:0	tr	tr	-	tr	tr
13 C 3-OH 12:0	tr	tr	-	tr	tr
14 C i 14:0	tr	tr	-	tr	tr
15 C ai 14:0	tr	tr	-	tr	tr
16 C 14:1	tr	0,609	-	0,453	tr
17 C 14:0	3,145	3,325	3,670	4,350	4,166
18 Ci 15:0	tr	tr	tr	tr	tr
19 C ai 15:0	tr	tr	tr	tr	tr
20 C 15:1	tr	tr	tr	0,047	tr
21 C 15:0	1,280	0,937	2,043	2,819	2,925
22 C 2-OH 14:0	tr	tr	tr	tr	tr
23 C 3-OH 14:0	0,111	tr	tr	tr	tr
24 C i 16:0	0,223	0,468	tr	1,262	0,157
25 C ai 16:0	tr	tr	tr	tr	tr
26 C 16:1 cis 9	4,314	7,588	6,719	2,800	6,826
27 C 16:1 trans 9				2,988	tr
28 C 16:0	52,159	52,697	50,013	51,123	47,772
29 C 2-OH 15:0	5,845	3,466	2,655	4,228	2,024
30 C 3-OH 15:0	1,865	1,311	0,899	1,113	1,868
31 C i 17:0	5,517	4,543	4,630	1,770	6,050
32 C ai 17:0	tr	0,703	tr	0,553	tr
33 C D 9, 10 17:0	4,566	1,920	3,599	0,483	2,322
34 C 17:1	1,559	1,358		1,287	1,650
35 C 17:0	2,614	0,843	1,778	2,483	2,404
36 C 2-OH 16:0	1,975	4,215	1,241	2,433	3,338
37 C 3-OH 16:0	tr	tr	0,727	0,141	tr
38 C i 18:0	1,252	2,201	tr	0,616	0,596
39 C ai 18:0	tr	tr	tr	tr	tr
40 C 18:2 cis 9,12	2,616	0,984	7,328	1,975	3,028
41 C 18:1 cis 9	3,807	6,323	10,849	11,066	6,776
42 C 18:1 cis 11	0,724		0,727		2,610
43 C 18:1 trans 9	1,308		0,803		1,581
44 C 18:0	0,807	2,201	tr	2,673	1,669
45 C Δ 9,10 19:0	1,141	0,562	tr	0,550	0,309
46 C 19:0	0,473	1,030	2,318	1,000	0,230
47 C i 20:0	tr	tr	tr	0,591	tr
48 C ai 20:0	tr	tr	tr	tr	tr
49 C 20:1	tr	tr	tr	tr	tr

TABLE 3
Continued

	A	B	C	D	E
50 C 20:0	tr		tr	tr	tr
Linear chain					
Saturated	70,170	71,664	64,452	70,436	67,668
Monounsaturated	11,711	15,878	19,098	18,641	19,443
Diunsaturated	2,616	0,984	7,328	1,975	3,028
Hydroxylated acids	9,796	8,992	5,522	7,915	7,230
Cyclopropane acids	5,707	2,482	3,599	1,033	2,631

tr = traces i = iso ai = anteiso

strains and their mutants. Thus the production of pigment does not seem to influence the principal metabolic activity and the differences are not related to total length of the carbon chain but only to the saturated/unsaturated ratio and to the total amount of lipid synthesized. FT-IR spectrometry and Gas Chromatographic analysis show a consistent variation both in the fatty acid content and in their distribution, indicating that the demand for acetate for the synthesis of the pigment might bring about lipid reduction and a greater membrane fluidity in the pigmented strains.
The slight decrease in total polysaccharides in the wild strains with respect to their non-pigmented mutants might also confirm the importance of the fate of the bicarboniose molecule in the process of pigment formation, taking into account that the carbon/nitrogen ratio has been found to influence the shift from production of pigment to that of polysaccharides (unpublished results).

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