JOURNAL OF BACTERIOLOGY, Feb. 1978, p. 811-815
0021-9193/78/0133-0811$02.00/0

Vol. 133, No. 2
Printed in U.S.A.

Comparative Studies of Porphyrin Production in *Propionibacterium acnes* and *Propionibacterium granulosum*

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Received for publication 25 August 1977

Porphyrin production by *Propionibacterium acnes* and that by *Propionibacterium granulosum* were compared. Porphyrin synthesized by both organisms was identified as coproporphyrin III on the bases of absorption and fluorescence spectra and behavior on paper chromatography and thin-layer chromatography. Quantitative, rather than qualitative, differences in production were found between these organisms. In general, *P. granulosum* produced significantly greater amounts ($P < 0.001$) of porphyrin than did *P. acnes*. δ-Aminolevulinic acid synthetase appeared to be the rate-limiting enzyme of the heme biosynthetic pathway in both organisms. The increased porphyrin production in *P. granulosum* is apparently associated with increased δ-aminolevulinic acid synthetase activity.

Tetrapyrrole-containing molecules, such as chlorophyll and heme, are widely distributed among various biological systems ranging from unicellular microorganisms to complex animal tissues. Genetic defects in porphyrin and heme metabolism have been reported in mutants of bacteria and yeasts (2, 4) as well as in hereditary diseases of porphyrin metabolism in man (16) in which there is prominent formation of intermediates of the porphyrin biosynthetic sequence.

Corynebacterium acnes, a normal microbial inhabitant of the skin, is responsible for the red fluorescence seen in sebum-rich human cutaneous follicular orifices when exposed to Wood's light (1), and this fluorescence was attributed to coproporphyrin III by Cornelius and Ludwig (3). The organism has been reclassified into the genus *Propionibacterium* and is divided into the species *P. acnes* and *P. granulosum* on the basis of selected biochemical tests (12, 20).

The present study was designed to determine whether these two organisms have any detectable differences in porphyrin metabolism and to assess selected biochemical changes in them that might account for the apparent differences in porphyrin production noted in the two species.

(This report was presented in part at the Third Annual Meeting of the American Society for Photobiology, Louisville, Ky., 22-27 June 1975.)

MATERIALS AND METHODS

Isolation and selection of porphyrin-producing strains. (i) Organisms. Colonies of *P. acnes* and *P. granulosum* were isolated from acne lesions of human subjects. They were suspended in 1% Triton X-100 solution and streaked out on Marshall-Kelsey agar plates to yield single-colony cultures.

Ten single-colony cultures were obtained. These colonies exhibited a wide spectrum of pigmentation, variously, white, pink, red, or brown. The six pigmented strains were identified as *P. granulosum*, whereas all four white strains were identified as *P. acnes*. Two representative strains, C_pa and C_pA, selected from *P. acnes* and *P. granulosum*, respectively, were used for further detailed studies of porphyrin production.

Identification as *Propionibacterium* was made on the bases of microscopic morphology, production of propionic acid, serum agglutination (Difco antiserum no. 554 or 605), and catalase production. Macroscopic morphology, gelatin liquefaction, and phage 174 susceptibility were used as criteria to differentiate *P. acnes* and *P. granulosum* (20).

(ii) Culturing the organisms. Thioglycolate (Difco Laboratories, Detroit, Mich.) or modified Marshall-Kelsey media (15) containing lactate and glucose as energy sources were used throughout these experiments unless stated otherwise. Cultures were enclosed in anaerobic jars (Baltimore Biological Laboratory, Cockeysville, Md.) containing a mixture of H_2 and CO_2 (95:5, vol/vol). For large-scale cell growth, cultures were grown semi-anaerobically in completely filled 2-liter screw-cap flasks at 35°C for 80 h in the low-glucose (0.2%) Marshall-Kelsey medium. Organisms were harvested by centrifugation for 10 min at 8,000 × *g* at 4°C; the cell pellet (washed and suspended in 0.05 M sodium phosphate buffer, pH 7.4) and supernatant solutions were used for comparative enzyme assays and porphyrin analyses, respectively. The dry weight of the organisms was calculated from a calibration curve by measurement of the turbidity at 675 nm (E_{675}) of cell suspensions (2).

Procedures for porphyrin analyses. (i) Extraction and separation of porphyrins. Methods used for extraction and analyses of porphyrins were based on those cited by Falk (7). Porphyrins were extracted separately from washed cell pellets and supernatant culture fluids with a mixture of ethyl acetate and glacial acetic acid (4:1, vol/vol) and then extracted into 1.5 N HCl for quantitative measurement.

In some experiments, the concentrated solution of porphyrins in the ethyl acetate layer was transferred to 3 N HCl, and further separation of uro-, copro- and protoporphyrins present in the 3 N HCl was based on their different partition coefficients between diethyl ether and three different concentrations of HCl solutions (4).

(ii) Identification of porphyrins. The extracted porphyrins were identified by the following criteria: (i) partition characteristics between ether and acidified aqueous solutions; (ii) absorption and fluorescence spectrophotometry; (iii) R_f values of methyl esters of the porphyrins (7), as determined by separation on thin-layer chromatography (Analtech, Inc., Newark, Del.; Silica Gel G, 250 μ) with a solvent system of benzene–ethyl acetate–methanol (85:13.5:1.5, vol/vol) (5); (iv) R_f values of free porphyrins on ascending paper chromatography with a solvent system of lutidine-water (10:3.4, vol/vol) in NH_3 atmosphere (6). The experimentally obtained spectra and R_f values were compared with those of known porphyrins and their respective porphyrin esters [Sigma Chemical Co., St. Louis, Mo.].

(iii) Measurement of porphyrins. Quantitative determinations of porphyrin concentration were measured fluorometrically as previously described (19), using a known concentration of coproporphyrin (CP) I solution in 1.5 N HCl as a standard. The values are expressed in terms of CP, since this was the major component of porphyrin mixtures.

Determination of ALA synthetase activity. (i) Preparation of cell-free homogenate. Cell suspensions at about 100 mg (wet weight)/ml of phosphate buffer (pH 7.4) were disrupted by ultrasonic disruption (Branson Sonifier) operated at maximal output for five 2-min periods with constant cooling. The cell wall debris was removed after centrifuging at 8,000 × g for 30 min in a Sorvall RC 2-B centrifuge and subsequently at 100,000 × g for 1 h in a Beckman model L ultracentrifuge to yield particulate and cytosol fractions, which were immediately used for assays of δ-aminolevulinic acid (ALA) synthetase. All preparations and assay procedures were performed at 4°C.

(ii) Colorimetric assay of ALA synthetase. The method of Granick (8), slightly modified, was used for determination of enzymatically formed ALA. Incubation mixtures contained: glycine, 100 mM; potassium succinate, 100 mM; tris(hydroxymethyl)amino-methane-hydrochloride (pH 7.4), 50 mM; sodium phosphate buffer (pH 7.4), 50 mM; sucrose, 100 mM; $MgCl_2$, 20 mM; ethylenediaminetetraacetate, 10 mM; ATP, 10 mM; glutathione (reduced form), 4 mM; pyridoxal 5'-phosphate, 0.267 mM; coenzyme A, 0.25 mM; succinic thiokinase (specific activity, 200 μmol of succinyl coenzyme A per h per mg), 100 μg; and enzyme preparation to a final volume of 2 ml. The ALA and aminoacetone pyrroles formed in the aqueous and ether phases were determined colorimetrically by reaction with modified Ehrlich's reagent according to the equations of Granick and co-worker (8, 17). The concentration of protein extracts was determined by the method of Lowry et al., using bovine plasma albumin standards (14).

RESULTS

Identification of porphyrins. Porphyrin was recovered from both supernatant fractions and cell pellets from cultures of the *P. granulosum* (C_pA) and *P. acnes* (C_pa) strains selected. Almost all of the porphyrin (95%) was found in the culture supernatant in either case.

The absorption maximum of the porphyrins produced by both strains was 403 nm (Soret band). Identical fluorescence spectra were obtained with emission maxima at 600 and 655 nm after excitation with 400-nm light. When these spectra were compared with various porphyrin standards on the bases of the position of the O'-O band maximum and the intensity ratio of the two peaks (1.25:1), an identity with the CP standard was apparent.

Separation as methyl esters on thin-layer chromatography provided confirmation that both strains contained, predominantly, CP. The porphyrin was identified as CP III by chromatographic isomer separation in 2,6-lutidine-water. Only one major fluorescent spot was present in all samples; however, traces of other porphyrins, including uro- and protoporphyrin, were detected on chromatography of porphyrins extracted from the supernatant solutions.

Effect of lactate and ALA on porphyrin production. Synthesis of porphyrin was investigated both in the lactate-containing Marshall-Kelsey medium and in a complex anaerobic medium (thioglycolate broth). Results are given in Table 1. Concentrations of extracellular porphyrins were noticeably higher in lactate-grown cells than in thioglycolate-grown cells. Porphyrin distribution patterns of both strains were, however, similar in that the major component formed in each was CP.

Whether or not porphyrin synthesis could be induced in the *P. acnes* strain was investigated by supplying the porphyrin precursor, ALA, in the growth medium. Patterns of CP development with age in Marshall-Kelsey medium supplied with various substrates are shown in Fig. 1A and B. Both strains exhibited pronounced increases in CP production by 40 h of growth and reached plateaus at 80 to 100 h of growth. Supplemental ALA stimulated porphyrin synthesis in low-glucose–lactate media in both strains, resulting in a 10- to 15-fold increase in *P. granulosum* and a 25- to 35-fold increase in *P. acnes* throughout the growth period. As

TABLE 1. *Comparison of total porphyrin and its distribution pattern between P. granulosum and P. acnes*[a]

Strain	Medium	Total porphyrin[b] (nmol/mg, dry wt)	Porphyrin distribution (%)		
			Uro[b]	CP	Proto[b]
P. granulosum	Lactate	4.68	8	87	5
	Thioglycolate	0.92	22	73	5
	Lactate + ALA[d]	40.86	34	52	14
P. acnes	Lactate	0.70	18	73	9
	Thioglycolate	0.18	15	78	7
	Lactate + ALA	20.21	21	70	9

[a] Cells were grown for 80 h in various media, and the centrifuged supernatant solutions were used for porphyrin study.
[b] Each value is the average of duplicate samples.
[c] Correction factors were used as compared to CP standard.
[d] Concentration of ALA used is 0.2 mmol/liter.

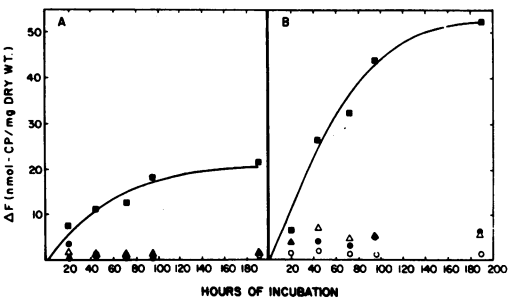

FIG. 1. *Effects of various growth media on porphyrin production by P. acnes (A) and that by P. granulosum (B). Media used were Marshall-Kelsey medium supplemented with (△) 0.2% glucose, (■) 0.2% glucose and ALA, (○) 2% glucose, and (●) 2% glucose and ALA. Porphyrin production shown is measured from the supernatant solution of cell cultures.*

shown in Table 1, the porphyrin distribution patterns characteristic for both strains were also maintained in synthesis from exogenous ALA, except for a relatively larger increase in uroporphyrin production. No noticeable increase of porphyrin production was observed in Marshall-Kelsey medium supplemented with either high or low concentrations of glucose alone or high glucose plus ALA. Addition of high concentrations (2%) of glucose to Marshall-Kelsey medium, either alone or with ALA, caused a more than twofold increase in cell mass. However, no increase of CP production was observed in the high-glucose–ALA medium to accompany this increasing cell growth. Since low-glucose-ALA enhanced CP production to a great extent, it appeared that a high concentration of glucose provided a marked inhibitor effect on ALA-induced porphyrin synthesis. Effects of these substrates on CP production in both strains at the stationary phase are shown in Table 2. The *P. granulosum* strain (C_pA) consistently demonstrated CP production higher than that of the

TABLE 2. *Effects of glucose and ALA upon porphyrin production in P. granulosum and P. acnes*[a]

Medium	Strain	CP[b] (nmol/mg, dry wt)	Standard deviation ($n = 3$)
0.2% glucose	*P. granulosum*	4.66	0.62
	P. acnes	0.68	0.16
0.2% glucose + ALA	*P. granulosum*	40.34	2.50
	P. acnes	20.16	1.22
2% glucose	*P. granulosum*	1.34	0.13
	P. acnes	0.18	0.05
2% glucose + ALA	*P. granulosum*	5.22	0.24
	P. acnes	0.23	0.08

[a] Cells were grown in the Marshall-Kelsey medium supplemented with glucose and ALA (0.2 mmol/liter).
[b] Calculated from triplicate samples of one experiment.

P. acnes strain (C_pa), approximately a 7-fold difference when grown in Marshall-Kelsey medium containing high or low concentrations of glucose and a 20-fold difference in high glucose and ALA. However, the disparity was reduced to only a 2-fold difference in low-glucose media when ALA was supplied.

Determination of ALA synthetase activity. As shown in Table 3, the ALA synthetase activity was predominantly associated with the cytosol fraction. Less than 5% of the total enzyme activity was found in the particulate fractions of cell-free homogenates. Specific activities of the crude preparations of ALA synthetase in the *P. granulosum* strain (C_pA) were three- to fivefold higher than those observed for the *P. acnes* strain (C_pa). In addition to differences noted in ALA synthetase activity, the amount of cytosol aminoacetones formed also varied between the two strains (0.86 and 0.15 nmol/mg of protein in C_pA and C_pa, respectively), sug-

TABLE 3. *Intracellular distribution of ALA synthetase in P. granulosum and P. acnes strains*[a]

Strain	Sp act of ALA synthetase[b] (nmol formed per h per mg of protein)		Enzyme activity in cytosol (%)
	Cytosol	Particulate	
P. granulosum	15	0.7	96
	13.8	0.4	97
	19.2	0.7	96
P. acnes	4.5	0.1	98
	4.0	<0.05	
	4.0	ND[c]	

[a] Cells were incubated for 80 h in Marshall-Kelsey medium supplemented with low glucose (0.2%).

[b] Data are obtained from three experiments, and each value is the average of duplicates.

[c] ND, Not determined.

gesting possible differences in the activities of other biosynthetic enzymes.

DISCUSSION

Two groups of *C. acnes,* reclassified as *P. acnes* and *P. granulosum,* differ in DNA guanine/cytosine ratio, indole production, ability to utilize certain nonfermentable sugars, and phage susceptibility (12, 20). These two organisms were studied for their comparative abilities to produce tetrapyrrole compounds.

We have demonstrated that CP III is the predominant porphyrin produced by both organisms on the bases of absorption and fluorescence spectrophotometric and chromatographic data. CP is the heme precursor most commonly accumulated in microorganisms (9, 13), especially in anaerobically grown cells, as maximal conversion of CP to protoporphyrin is an oxygen-dependent step (10, 11, 18). Investigations of porphyrin and heme biosynthesis in *Propionibacterium* cells grown on complex and semidefined media have shown that porphyrin biosynthesis is influenced by environmental conditions. This conclusion is derived from the evidence that various growth media are able to promote or to suppress total porphyrin synthesis as well as to alter the distribution pattern of individual porphyrins within the biosynthetic sequence.

Although there were pronounced quantitative differences in their abilities to form porphyrin under various growth conditions, *P. granulosum* consistently demonstrated a greater CP production than *P. acnes.* We have some evidence (unpublished data) indicating that the CP-accumulating *P. granulosum* has a lower level of catalase activity than *P. acnes.* These preliminary data suggest that the prophyrin-heme pathways operative in these two strains may differ in functional emphasis, which appears accentuated toward the accumulation of porphyrin in *P. granulosum* cells, whereas in *P. acnes,* the formation of a heme derivative (catalase) appears emphasized.

Many studies have addressed the regulatory mechanism controlling the metabolic balance between the biosynthesis of porphyrins and hemes. Without controls, cells overproduce nonfunctional heme precursors. In the present study, the ALA synthetase step in the heme biosynthetic pathway was bypassed by adding ALA to the growth medium. The exogenous ALA was then rapidly converted to porphyrin, resulting in marked increases in CP levels in both strains. It may be concluded, in analogy to other well-studied biological systems, that ALA synthetase is the rate-limiting enzyme of the heme biosynthetic pathway in these two strains. Enzymes in the pathway beyond ALA synthetase could be induced to relatively nonlimiting levels. The difference in the ability of the two species to produce CP was minimized when ALA was added to the growth medium. This suggests that the major biochemical difference between *P. granulosum* and *P. acnes* occurs before ALA formation, due to disparity either in availability of ALA precursors or in activity of ALA synthetase. Our finding that ALA synthetase activity in *P. granulosum* is more than threefold greater than that of *P. acnes* correlates well with the greater ability of *P. granulosum* to form CP. However, it should be noted that activities of other porphyrin-related enzymes might also be unequal, as indicated by the twofold differences in CP production between the two strains observed even with excess ALA supplied.

ACKNOWLEDGMENTS

We thank C. H. Zierdt (National Institutes of Health) for the supply of phage 174 and the microbiology laboratory at the College of Physicians and Surgeons, Columbia University, for carrying out the gas-liquid chromatographic analysis of the fatty acids.

This study was supported by Public Health Service (PHS) grant CA-13696 from the National Cancer Institute and PHS research grant AM-18549 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

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