COMPARATIVE TAXONOMY OF CRYSTALLOGENIC STRAINS OF *PSEUDOMONAS AERUGINOSA* AND *PSEUDOMONAS CHLORORAPHIS*

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ABSTRACT

HAYNES, WILLIAM C. (Northern Utilization Research and Development Division, Peoria, Ill.) AND LENORA J. RHODES. Comparative taxonomy of crystallogenic strains of *Pseudomonas aeruginosa* and *Pseudomonas chlororaphis*. J. Bacteriol. **84**:1080–1084. 1962—Only 11 of 39 strains received in the Agricultural Research Service Culture Collection under the designation *Pseudomonas chlororaphis* proved to be authentic; 28 were typical, pyocyanogenic strains of *P. aeruginosa*. The reason for this disproportionately high rate of misidentification apparently arises from an erroneous belief that the ability to produce green and yellow crystals of chlororaphin and oxychlororaphin is confined to *P. chlororaphis*. The ability of many strains of *P. aeruginosa* to do likewise is not well known. Inasmuch as the characteristic is not unique to *P. chlororaphis*, other criteria are required to distinguish crystallogenic strains of these species. After a taxonomic comparison of 18 strains of *P. chlororaphis* and 47 crystallogenic strains of *P. aeruginosa*, it was determined that there are three main distinctions: (i) *P. aeruginosa* grows well at 42 C but fails to grow upon serial transfer at 5 C, whereas *P. chlororaphis* fails to grow at 42 C, but grows well at 5 C; (ii) most strains of *P. aeruginosa* produce pyocyanin, whereas *P. chlororaphis* strains do not; (iii) *P. aeruginosa* cells possess only one or two polar flagella, whereas *P. chlororaphis* usually has at least four, sometimes as many as eight, polar flagella.

Only 11 of 39 strains received in the Agricultural Research Service Culture Collection under the designation *Pseudomonas chlororaphis* (the name *P. chlororaphis* is used in this paper for convenience and in conformity with current usage) conformed to the description given by Breed, Murray, and Smith (1957). The other 28 were typical, pyocyanogenic strains of *P. aeruginosa*. In seeking an explanation for the apparent confusion, we concluded that the ability of *P. aeruginosa* to produce green crystals of chlororaphin in a variety of media is not widely known. This property, once considered unique for *P. chlororaphis*, is now known to be a characteristic of many, if not all, strains of *P. aeruginosa* (Birkofer, 1947; Sierra and Veringa, 1958; Takeda, 1958). These considerations led us to realize the need for wider dissemination of information about the crystallogenic propensities of both species and to search for characteristics that would distinguish between them. To these ends we characterized and compared all available strains of *P. chlororaphis* and crystallogenic strains of *P. aeruginosa*.

MATERIALS AND METHODS

Crystallogenic strains of *P. aeruginosa* (47) and *P. chlororaphis* (18) were characterized and compared. So that the 28 strains of *P. aeruginosa* that are masquerading as *P. chlororaphis* may be recognized, their histories are given in Table 1. The histories of 18 authentic strains of *P. chlororaphis* are presented in Table 2. Only three were listed in catalogues of ready availability.

Methods used were those prescribed by the Society of American Bacteriologists (1957), except that the oxidase test was performed as recommended by Kovacs (1956), gluconate oxidation and observation of slime production were carried out in the media of and by methods suggested by Haynes (1951), and pyocyanin formation was determined in Burton's (Burton, Campbell, and Eagles, 1948) medium in stationary cultures at 28 C. A presumptive test for pyocyanin (Wetmore and Gochenour, 1956) was performed at 4-day intervals, or whenever the color of the culture suggested pyocyanin might be present.

Temperature relationships were determined in the following manner. A young culture grown from stock at 28 C was used to inoculate a Tryp-

TABLE 1. *Histories of accessions labeled Pseudomonas chlororaphis that proved to be crystallogenic strains of P. aeruginosa*

NRRL no.	Year received	Donor and no.	Remarks
B-743	1948	R. Y. Stanier as A. 3.30	
B-937	1949	M. P. Starr as M.D. 44.1	Isolated from guayule rets
B-1684	1955	M. B. Morris as B-60	Isolated from compost
B-1731	1956	NCIB as 8730	From C. B. van Niel as M.D. 44.1; isolated from guayule rets as strain 938
B-1735 to B-1753	1956	J. O. Harris	Isolated from failing and nonfailing sections of asphalt highway (Harris, 1956)
B-1853	1957	P. V. Liu as M.D. 44.3	From G. Knaysi as strain 982 from guayule rets
B-1948	1957	P. Murray as M.D. 44.1	Isolated from guayule rets
B-1954	1957	P. Murray as M.D. 44.2	Strain 967 from guayule rets
B-2579	1960	M. P. Starr as 2384	From Hutner
P-915[a]	1960	M. P. Starr as 2385	From C. B. van Niel as M.D. 44.1; therefore identical with B-937, B-1731, and B-1948

[a] A temporary number signifying a culture not retained in the Collection.

TABLE 2. *Histories of authentic Pseudomonas chlororaphis strains in the ARS Culture Collection*

NRRL no.	Year received	Donor and no.	Remarks
B-560 B-561	1943	NRRL isolates	Plate contaminants
B-977	1949	NCTC as 7357	
B-1095 B-1097 B-1098	1950	NRRL isolates	Isolated from soil
B-1541	1954	E. T. Eltinge	Isolated from soil
B-1632	1954	W. J. Dowson as 261	
B-1682	1955	NRRL isolates	Isolated from frass from Engelman spruce tree
B-1854	1957		Isolated from soil
B-1869 B-1870 B-1878 to B-1881	1957	J. D. Stout as K7, H26, XXXVIIIa, XXXVIIIb, H25, and H32, respectively	Isolated from soil
B-2075	1958	O. Lysenko as 4	Isolated from a caterpillar
B-2266	1959	A. Fuchs	

tone-glucose-yeast extract agar slant (Haynes, Wickerham, and Hesseltine, 1955), which then was incubated in a water bath at the desired temperature until good growth developed. A strain was considered incapable of growth at a given temperature unless it could survive at least three serial transfers at that temperature, after acclimation. Cultures were acclimated by serial transfer at a lower or higher temperature, depending upon whether temperatures above or below 28 C were being investigated.

We were unable to find a medium in which we

could elicit either chlororaphin or oxychlororaphin production, or both, at will. However, crystals were formed by one or more strains in almost every medium used in diagnostic tests. Those in which crystals were most often seen, in order of frequency, were gelatin stabs, Tryptone-glucose-yeast extract agar slants, and Fuchs' agar (1956).

Flagella were stained by Leifson's (1951) method.

Colony morphology on Fuchs' sucrose agar was observed in petri plates which had been spot-inoculated and then incubated at 28 C.

Each test and observation was repeated at least once, usually several times, over a period of years. Thus, the constancy and reliability of results could be evaluated.

RESULTS

Besides the ability to form green crystals of chlororaphin or yellow crystals of oxychlororaphin, or of both, crystallogenic strains of *P. aeruginosa* and *P. chlororaphis* have many characteristics in common. All are gram-negative rods that oxidize potassium gluconate to a reducing compound presumed to be 2-ketogluconate; liquefy plain and nutrient gelatin; and cause an alkaline reaction in milk, coagulate it, and then digest the curd. They also reduce the litmus in litmus milk, reduce nitrates to nitrites or beyond, fail to attack cellulose or to form indole, and give a positive Kovacs' oxidase test. The medium in cultures of most strains of both species, whether solid or liquid, becomes varicolored because of the production of different colored pigments which are soluble. The color may be yellow, brown, greenish yellow, green, bluish green, or blue. Some *P. aeruginosa* cultures may be red, purple, and even black. It is never safe to rely on the color of the medium as an aid to identification. As will be pointed out below, however, certain extractable pigments are dependable aids. Also, many pigment-producing strains of *P. aeruginosa* and *P. chlororaphis* are fluorescent. A heavy slime is produced by most strains in stationary culture in the same gluconate medium as that used for study of gluconate oxidation.

The characteristics by which strains of *P. aeruginosa* and *P. chlororaphis* may be distinguished are shown in Table 3 and are as follows.

Flagellation. Flagellated strains of *P. aeruginosa* possess one, sometimes two, polar flagella, whereas flagellated strains of *P. chlororaphis* possess at least four, sometimes eight, polar flagella.

TABLE 3. *Differential characteristics of crystallogenic strains of Pseudomonas aeruginosa and P. chlororaphis*

Characteristic		No. of strains	
		P. aerugi-nosa	*P. chloro-raphis*
No.[a] of polar flagella	None	2	1
	1	21	0
	2	24	0
	4–8	0	17
Pyocyanin	Formed	38	0
	Not formed	9	18
Color of growth	Varicolored[b]	47	0
	Flesh-colored to yellow	0	17
Ability to survive serial transfer	42 C	47	0
	39 C	47	0
	37 C	47	11[c]
	35 C	47	18[d]
	28 C	47	18
	15 C	47	18
	5 C	0	18
Colonies on Fuchs' agar	Not mucoid	47	3
	Mucoid	0	15

[a] Largest number of flagella repeatedly observed.

[b] Translucent growth takes on color of the underlying medium.

[c] These strains often failed to survive serial transfer at 37 C.

[d] Six strains required adaptation to grow at 35 C; two others varied in response.

Pyocyanin production. One of the extractable pigments referred to above is pyocyanin. It is a blue, water-soluble phenazine pigment which is easily extracted with chloroform from cultures of most strains of *P. aeruginosa.* It is not produced by *P. chlororaphis* or by any other microorganism so far as is known. Although Burton's medium is mentioned specifically as the medium of choice for evoking pyocyanin formation, several other media were also used in arriving at the conclusion in each instance that pyocyanin was or was not formed.

Another medium-soluble, chloroform-extractable pigment which may be present in *P. aeru-*

ginosa cultures is phenazine α-carboxylic acid (Takeda, 1958). However, it is not known yet whether the presence of this pigment would be helpful in identification and classification of *P. aeruginosa*. Phenazine α-carboxylic acid is produced by *P. aureofaciens* (Kluyver, 1956; Haynes et al., 1956) and is a diagnostic characteristic for it.

Insoluble pigment production. The growth of *P. chlororaphis* on solid media is often flesh-colored to yellow, in contrast to that of *P. aeruginosa* which, being translucent, takes on the color of the underlying medium.

Temperature relationships. The most reliable differential characteristics involved temperature relationships of growth. It is a well-known ability of *P. aeruginosa* to grow almost as well at 42 C as at 37 C. All crystallogenic strains of *P. aeruginosa* conformed to this description. None of our strains of *P. chlororaphis* was able to grow at 42 C (Table 3). In fact, none of them was able to grow at 39 C. At 37 C, 11 strains occasionally were able to grow, but could not be adapted to survive serial transfer consistently. Even at 35 C, six strains, those from New Zealand, were reluctant to grow, but they could be adapted. Two others gave erratic results.

All strains of *P. chlororaphis* grew at 15 and 5 C. All strains of *P. aeruginosa* grew at 15 C but failed to survive serial transfer at 5 C. Occasionally, a strain of *P. aeruginosa* would grow initially at 5 C, but none of them survived serial transfer at 5 C.

Character of growth on sucrose agar. Another differential characteristic concerns the character of growth on Fuchs' (1956) 4% sucrose agar. Colonies of *P. aeruginosa* strains were invariably thin, flat, translucent, and spreading. Three strains of *P. chlororaphis* (B-1097, B-1098, and B-1682) produced growth indistinguishable from that of *P. aeruginosa*. Most of the *P. chlororaphis* strains (15) produced thick, convex, mucoid, opaque colonies, presumably because they produced copious amounts of levans (Fuchs, 1956, 1959).

DISCUSSION

Birkofer (1947) was the first to recognize that strains of *P. aeruginosa* produce crystals of chlororaphin. His report apparently was unseen or discounted; there are few references to it in the literature. Sierra and Veringa (1958*a*, *b*) and Takeda (1958), working independently, were the first investigators to confirm Birkofer's findings. They, however, did not seem to realize that the ability to produce chlororaphin was widespread among *P. aeruginosa* strains, or that it might lead to confusion with strains of *P. chlororaphis*. Crystallogenic strains of *P. aeruginosa* and of *P. chlororaphis*, however, have many characteristics in common, and the differences between them are readily missed. Differences in flagellation, for instance, are not well known. The presence of pyocyanin sometimes goes undetected because of an inappropriate medium or incubation temperature or because its blue color is masked or modified by the presence of other soluble pigments, so that chloroform extraction is not carried out. Furthermore, some strains (in this case, 20%) do not produce pyocyanin. Temperature relationships, the most reliable differential characteristics, are seldom investigated and, to our knowledge, levan formation on Fuchs' medium has not previously been used as a diagnostic property of these species. Thus, the similarities have sometimes been more obvious than the differences.

The quickest and simplest way to identify a green or yellow crystal-forming pseudomonad is to check for ability to grow at 42 C. If growth occurs, *P. aeruginosa* is the correct identity. If growth fails, *P. chlororaphis* is correct. If further confirmation is desired, one of the other differential characteristics may be determined. They require appreciably longer periods of time for their determination and will usually not be necessary.

We were unsuccessful in devising a medium in which crystallogenic strains of *P. aeruginosa* and *P. chlororaphis* would consistently produce crystals of chlororaphin and oxychlororaphin. Therefore, it is obvious that *P. chlororaphis*, whose specific identity is dependent upon observation of crystals, will be virtually unrecognizable if crystals are never seen. Such strains would probably be taken for *P. fluorescens*.

Aside from the ability to produce crystals of chlororaphin and oxychlororaphin, crystal-forming strains of *P. aeruginosa* are indistinguishable from acrystallogenic strains. It is likely that many, if not most, strains of *P. aeruginosa* may be induced to form colored crystals. In a survey of 17 stock strains of *P. aeruginosa*, 9 not previously known capable of producing crystals did so. Colored crystals are often late in appearing in cultures, and are perhaps often missed because cultures are discarded too soon.

For study of the temperature relationships of pseudomonads, incubation temperatures of 37

and 42 C have come into fairly general use. The value of incubation at 42 C in diagnosing *P. aeruginosa* is beyond question, but, for pseudomonads that fail to grow at this temperature, we believe 37 C is too close to the maximal growth temperature of some of them to be taxonomically useful. For instance, the growth of 11 strains of *P. chlororaphis* that sometimes survived serial transfer at 37 C was poor. We hoped for a more clear-cut result by use of either 35 or 39 C. At 39 C, all 18 strains of *P. chlororaphis* failed to survive serial transfer. At 35 C, ten grew well, six grew poorly or sporadically but could be adapted to good growth, and two showed variable growth but could not be adapted. (It is interesting to note that the six that were adaptable to growth at 35 C came from New Zealand.) Apparently, 35 C is close enough to the maximum that a few strains still respond erratically, but results are better than at 37 C.

The sum of these results is that there is a clear-cut difference in the temperature relationships of the two crystallogenic pseudomonads. All strains of *P. aeruginosa* grew at 42 C, whereas all strains of *P. chlororaphis* failed to do so. Furthermore, no strain of *P. chlororaphis* could be adapted to grow at temperatures above 37 C, and even at this temperature none of them could survive serial transfer consistently. Likewise, at 5 C, all strains of *P. chlororaphis* survived serial transfer, whereas none of the *P. aeruginosa* strains could be induced to do so.

In the course of studying the adaptability of strains to grow in the vicinity of their maxima, it was observed that some strains which could initiate good growth at a given temperature showed increasing reluctance to do so with each serial transfer at that temperature. This phenomenon was noted with *P. chlororaphis* strains at 37 and 35 C, and with *P. aeruginosa* at 5 C. We believe that a strain should survive at least three serial transfers at a given temperature before being characterized for taxonomic purposes as able to grow at that temperature. In cases in which the amount of growth diminished with each successive transfer at a given temperature, we continued transferring serially until it was clear that the microorganism would not adapt.

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