ACETIC ACID PRODUCTION FROM ETHANOL BY FLUORESCENT PSEUDOMONADS

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Apart from the observation by Alsberg (1911) that gluconic acid is produced from glucose by *Phytomonas savastanoi*, the oxidative metabolism of fluorescent pseudomonads has received little attention until very recently. For the most part it has been tacitly assumed that these organisms, by virtue of their obligately aerobic nature, carry out a complete mineralization of organic substrates (den Dooren de Jong, 1926). However, the researches of Pervozvanski (1939*a*, 1939*b*), followed by those of Lockwood *et al.* (1941, 1946), have established the unexpected fact that the dissimilation of monosaccharides by the majority of fluorescent pseudomonads is accompanied by the production and accumulation of the corresponding hexonic or pentonic acids in large amounts. Some strains when acting on glucose also carry the oxidation further to 2-ketogluconic acid (Pervozvanski 1939*a*, Lockwood *et al.* 1941) and α-ketoglutaric acid (Lockwood and Stodola, 1946). The failure of all previous investigators except Alsberg to observe these phenomena may be ascribed to the use of weakly buffered and insufficiently aerated media (e.g., the customary tubes of carbohydrate broth), since acidity and poor oxygen supply are both limiting factors for the transformations in question.

The oxidation of monosaccharides to the corresponding -onic acids is a pattern of biochemical behavior that occurs elsewhere among bacteria, so far as is at present known, only in the *Acetobacter* group. Coupled with the frequently overlooked morphological similarities, it serves, as Vaughn (1942) has pointed out, to indicate a close relationship between the genera *Acetobacter* and *Pseudomonas*. Consequently it seemed of interest to find out whether the primary biochemical characteristic of the acetic acid bacteria, namely the oxidation of ethanol to acetic acid, might not also exist in the genus *Pseudomonas*.

MATERIALS AND METHODS

Thirteen strains of fluorescent pseudomonads were studied, of which one was a strain of *Pseudomonas aeruginosa* and the remainder belonged to the *Pseudomonas fluorescens* species-group.[1] Three cultures (designated by the prefix NRRL) were received from Dr. Lockwood, by whom they had been used in studies on the metabolism of monosaccharides. The others (designated by the prefix A.3.) were isolated locally from soil, using the customary enrichment methods (den Dooren de Jong, 1926).

[1] The term "*P. fluorescens* species-group" is used to designate pseudomonads producing a fluorescent pigment but devoid of accessory phenazine pigments (pyocyanin, chlororaphin, etc.). The taxonomic criteria in current use with this group are inadequate, in my opinion, to justify any further specific subdivisions.

Ability to use ethanol as sole carbon source was tested by streaking on mineral agar plates (0.1 per cent NH_4Cl, 0.1 per cent K_2HPO_4, 0.05 per cent $MgSO_4$, and 1.5 per cent agar) containing 1.0 per cent ethanol, and comparing growth with that on a control plate devoid of carbon source. Preliminary observations on acid production from ethanol were made by streaking on mineral or peptone agar plates containing ethanol and $CaCO_3$ and noting the formation of cleared zones in the carbonate around the bacterial growth. This method is also extremely useful for a rough screening of strains that produce acid from sugars.

For quantitative studies on ethanol oxidation, the organisms were grown in 250-ml Erlenmeyer flasks containing 50 ml of medium. Incubation was at 30 C on a shaking machine. The medium consisted of 0.5 per cent Difco peptone with various concentrations of ethanol and, in some experiments, also 0.5 per cent $CaCO_3$.

TABLE 1

Acetic acid production from ethanol by strains of the P. fluorescens species-group after 5 days

STRAIN	RESIDUAL ETHANOL	ETHANOL USED	ACETIC ACID FORMED	YIELD OF ACETIC ACID*
	mg	*mg*	*mg*	
Uninoculated	536			
A.3.1	82	454	58	10
A.3.2	0	536	242	35
A.3.3	21	515	145	21
A.3.6	0	536	313	45
A.3.8	152	384	365	71
A.3.9	0	536	45	6
A.3.10	48	488	71	11
NRRL B-13	24	512	5	1

Medium: 0.5 per cent peptone, 0.5 per cent $CaCO_3$, and 1.0 per cent ethanol.
* Expressed as percentages based on ethanol oxidized.

Ethanol was determined by dichromate oxidation of neutral distillates and estimation of residual dichromate; acetic acid, by titration of steam distillates. The acetic acid was identified by the iodine-lanthanum reaction and by formation of the characteristic copper salt (Meyer, 1933, p. 101).

RESULTS

Nine of the 13 strains were capable of developing abundantly on mineral, ethanol agar with ethanol as the sole carbon source. The remaining 4 (including two—NRRL B-14 and B-25—received from Dr. Lockwood) failed to develop on this medium. Of the 9 positive strains, 7 produced sufficient acid on mineral, ethanol, $CaCO_3$ agar to cause a marked dissolution of the carbonate, and one more (NRRL B-13) produced a very slight amount of acid. The only ethanol-utilizing strain which failed to produce any acid whatsoever was the isolate of *P. aeruginosa*. Ethanol also gave rise to acid production when the mineral base

was replaced by 0.5 per cent peptone; indeed, under these conditions slightly more acid appeared to be formed.

Quantitative data on ethanol oxidation and acetic acid formation by the 8 acid-producing strains are shown in table 1. The medium contained 0.5 per cent $CaCO_3$ and slightly over 1 per cent ethanol. It can be seen that the degree of acetification varies very greatly from strain to strain. Some carry out a virtually complete oxidation of the ethanol with negligible accumulation of

TABLE 2

Total titratable acidity and final pH produced by three strains of fluorescent pseudomonads when grown in 50 ml of peptone, 1.5 per cent ethanol broth

STRAIN	TITRATABLE ACIDITY, ML OF 0.1 N			FINAL pH
	36 hr	60 hr	84 hr	84 hr
Uninoculated	2.0	2.0	2.0	7.35
A.3.1	1.5	8.5	8.5	4.55
A.3.2	1.3	10.1	10.1	4.50
A.3.8	7.0	7.0	7.0	4.95

Cultures grown at 30 C on a shaking machine.

TABLE 3

Acetic acid production from ethanol by strain A.3.8

MEDIUM*	FINAL pH	TITRATABLE ACIDITY ML of 0.1 N	RESIDUAL ETHANOL	ETHANOL USED	ACETIC ACID FORMED
			mg	*mg*	*mg*
Peptone, control	7.15	1.6			
Peptone, inoculated	8.80	0.0			
Peptone, ethanol, control	7.15	1.8	535		
Peptone, ethanol, inoculated	4.55	10.0	276	259	50
Peptone, ethanol, $CaCO_3$, control	7.75		555		
Peptone, ethanol, $CaCO_3$, inoculated	5.00		57	498	231

Cultures were grown for 5 days at 30 C on a shaking machine.

* Constituents of the medium were used in the following concentrations: peptone, 0.5 per cent; ethanol, 1.0 per cent; and $CaCO_3$, 0.5 per cent.

acetic acid, whereas others convert a substantial proportion of the ethanol oxidized into acetic acid.

In the absence of $CaCO_3$, acid production (as gauged by titratable acidity) is slight, even with the most actively acetifying strains. This is owing to the fact that the pH soon drops below 5.0 and the organisms die off. Typical figures for titratable acidities and final pH in peptone ethanol broth cultures are given in table 2. Streaked plates made from such cultures after 3 to 4 days reveal the presence of very few viable cells. A somewhat more detailed picture of the effect of $CaCO_3$ addition is given for strain A.3.8. in table 3.

DISCUSSION

The present demonstration that some fluorescent pseudomonads can produce substantial amounts of acetic acid from ethanol might have been predicted in the light of recent work on their metabolism. Although acetification is not a universal property of these organisms, some strains being unable to attack primary alcohols at all, its very existence in the *P. fluorescens* species-group raises a nice taxonomic problem, since the family *Acetobacteriaceae* and the genus *Acetobacter* are currently segregated from other pseudomonads primarily on the basis of their ability to produce acetic acid from ethanol. In view of the extensive morphological and biochemical parallelism between acetic acid bacteria and organisms of the *P. fluorescens* type, it seems indefensible any longer to maintain a family *Acetobacteriaceae*; its members should be incorporated in the family *Pseudomonadaceae*. The genus *Acetobacter*, if it is to be kept at all, must be redefined in a manner which no longer stresses so exclusively the fact of acetification. As an additional differential property, acid tolerance, which is so marked in these organisms as contrasted with other heterotrophic pseudomonads, should be considered.

SUMMARY

Certain strains of the *Pseudomonas fluorescens* species-group can oxidize ethanol with the production and accumulation of acetic acid. The intensity of acetification varies greatly from strain to strain. Acetification proceeds best in a medium well buffered with calcium carbonate. In poorly buffered media, ethanol oxidation is soon checked by increasing acidity.

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