NUTRITION AND PHYSIOLOGY OF *PSEUDOMONAS FRAGI*[1]

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Some observations of the nutritional requirements of *Pseudomonas fragi*, a psychrophilic organism implicated in spoilage of dairy products (fruity aroma), have been published. Omelianski (1923) claimed that aroma production was dependent upon the presence of nitrogenous organic matter and Hussong *et al.* (1937) studied the biochemical characteristics of *P. fragi* and observed its growth on complex media. However, neither of these reports concerned the nutritional requirements of *P. fragi* in chemically defined media. The present work was a study of the nutritional needs for growth and the substrates essential for aroma production by 6 strains of *P. fragi*.

MATERIALS AND METHODS

Cultures. Six strains of *P. fragi* were studied, 5 of which were isolated at the University of Connecticut. Strain ISC was obtained from the Dairy Industry Department, Iowa State College. All cultures conformed in all respects to the description in *Bergey's Manual* (Breed *et al.*, 1948) and were maintained on cystine trypticase agar stabs at 4 C.

Glassware and test media. All glassware was treated with $Na_2Cr_2O_7 \cdot H_2SO_4$ cleaning solution and thoroughly rinsed before use. Kimble screw cap tubes closed with linerless caps were used.

A salt solution of the following composition in g/L was a satisfactory source of inorganic requirements: K_2HPO_4, 3.0; NaCl, 5.0; $FeSO_4$, 0.0002; $CaCl_2$, 0.0002; $MgSO_4$, 0.0002. In the study of carbon sources, NH_4Cl, 4.0 mg/ml, was added as a nitrogen source. Optimum C concentration was 2.0 mg/ml when the naturally occurring isomers were tested. When nitrogen compounds were studied, glucose, 5.0 mg/ml, was added as a carbon source. Optimum N concentration was 0.140 mg/ml. All experimental media were adjusted to pH 7.5 and sterilized by filtration through ultrafine sintered glass. Five ml quantities of each test medium were dispensed into sterile tubes and incubated to insure sterility.

Culture conditions. In nutrient broth (pH 7.5) incubated at 20 C and shaken at about 100 excursions/min for 18 hr, good growth was obtained. Accordingly, these culture conditions were used throughout, except that the incubation period was extended to 48 hr to compensate for the slower growth in all test media.

Test method. One loopful of such nutrient broth cultures served as the inoculum for the first transfer into experimental media. Three successive loopwise transfers were made in the test media at 48 hr intervals. Growth was measured photometrically with the Lumetron model 400A, using the blue filter (420 mμ). The readings obtained for the third and fourth transfers compared closely and therefore the average of these readings was used in establishing the suitability of a carbon or nitrogen compound. A turbidity (100-light transmittance) of 20 per cent was considered to represent a good growth response.

Compounds studied. Sixty-two compounds were tested for their ability to support growth when supplied as sole sources of carbon. The compounds were of the following classes: pentoses, hexoses, disaccharides, trisaccharides, polyhydric alcohols, monohydric alcohols, amino acids, fatty acids, and various metabolic intermediates. In the study of nitrogen sources, 30 compounds including amino acids, purines, pyrimidines, urea, $NaNO_2$, $NaNO_3$, $(NH_4)_2SO_4$ were substituted singly for NH_4Cl. The single compounds which had been found to be suitable carbon and nitrogen sources were then incorporated into experimental media as sole sources of both elements.

All cultures were examined organoleptically to detect substrates which served as precursors to the aroma compound(s).

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RESULTS AND DISCUSSION

Some degree of heterogeneity was observed in the 6 test strains. In general strains $1M_1$ and ISC appeared to be somewhat more fastidious than the other strains. However, this variation is not considered extensive enough to be the basis for taxonomic differentiation between the isolates studied. The results indicate that the nutrition of *P. fragi* is similar to that of other pseudomonads in its ability to grow in the absence of exogenous vitamins when an inorganic nitrogen compound and a carbon source are supplied.

Carbon compounds studied. Eighteen of the 62 carbon compounds supported good growth of at least 1 of the 6 test strains. These results are summarized in table 1. These compounds included glucose, ribose, gluconolactone, glycerol, amino acids, and metabolic intermediates associated with glucose dissimilation and the tricarboxylic acid cycle. The fact that ribose and gluconolactone were utilized provides some evidence for a mechanism for glucose metabolism which appears to be characteristic of pseudomonads. *Pseudomonas saccharophila* (Entner and Doudoroff, 1952), *Pseudomonas lindneri* (Gibbs and DeMoss, 1954), *Pseudomonas aeruginosa* (Norris and Campbell, 1949), and *Pseudomonas fluorescens* (Entner and Stanier, 1951) have been shown to utilize enzymatic pathways alternate to the classical Embden-Meyerhof-Parnas system. In addition, Kovachevich and Wood (1955) have demonstrated 6-phosphogluconate dehydrase in sonic extracts of *P. fragi* and Lockwood *et al.* (1941) reported the conversion of glucose to 2-ketogluconic acid by growing cells of *P. fragi*.

Krebs cycle intermediates including acetate, citrate, α-ketoglutarate, succinate, fumarate, malate, and oxalacetate served as good carbon sources. In addition, compounds associated with the tricarboxylic acid cycle such as pyruvate, lactate, alanine, and glutamic acid were suitable carbon sources.

TABLE 1

Utilization of 18 carbon sources by 6 strains of Pseudomonas fragi in a salts medium

Carbon Compounds	Strain					
	$1S_4$	$1H_1$	$1CH_7$	$1CH_6$	$1M_1$	ISC
Ribose	40*	7	7	13	9	4
Glucose	60	44	41	45	17	26
Glycerol	28	19	24	25	0	13
L-Histidine·HCl	68	46	55	50	44	51
L-Arginine·HCl	30	26	29	42	31	33
L-Glutamic acid	50	39	35	41	34	46
DL-Alanine	10	6	6	5	3	20
L-Proline	31	25	29	15	18	28
Sodium pyruvate	59	58	64	55	59	64
Sodium acetate	40	24	3	1	2	1
Citric acid	50	35	40	41	39	42
Succinic acid	49	37	4	0	0	0
α-Ketoglutaric acid	36	29	35	33	29	42
Fumaric acid	47	24	0	0	0	0
L-Malic acid	60	35	54	55	39	56
Oxalacetic acid	32	30	35	29	39	43
Lactic acid	36	29	33	36	29	32
Gluconolactone	49	39	42	59	40	42
Control nutrient broth	63	59	63	52	43	52

* Figures represent average turbidities (100 − light transmittance) of third and fourth transfers in test media.

TABLE 2

Biacetyl formation by six strains of Pseudomonas fragi in an inorganic medium containing four compounds as sole carbon sources

Carbon Source	Strain					
	$1S_4$	$1H_1$	$1CH_7$	$1CH_6$	$1M_1$	ISC
Sodium pyruvate	+*	+	+	+	+	+
Fumaric acid	−	+	−	−	−	−
Malic acid	+	+	+	+	+	+
Oxalacetic acid	−	+	+	+	−	+

* Strong odor of biacetyl and positive Voges-Proskauer test. (Levine *et al.*, 1934.)

Additional evidence for the Krebs cycle in the test strains was found during the study of carbon compounds. A buttery aroma suggestive of biacetyl was detected in cultures of *P. fragi* when fumarate, malate, oxalacetate, and pyruvate were supplied as the sole carbon source. Subsequent Voges-Proskauer tests (Levine *et al.*, 1934) indicated that biacetyl was produced from these substrates (table 2). Since the formation of biacetyl involves pyruvate as an intermediate, it is clear that fumarate, malate, and oxalacetate are readily converted to pyruvate. The conversion of fumarate and malate may proceed by way of reactions known to occur in the Krebs cycle. From these data it would appear that at least two enzymatic processes are possible in the metabolism of pyruvate by *P. fragi*, namely,

TABLE 3

Utilization of 28 nitrogen sources by 6 strains of Pseudomonas fragi in a glucose-salts medium

Nitrogen Compounds	Strain					
	$1S_4$	$1H_1$	$1CH_7$	$1CH_6$	$1M_1$	ISC
L-Arginine·HCl	30*	31	39	42	35	35
L-Histidine·HCl	41	25	41	39	41	39
L-Lysine·HCl	31	23	35	25	27	27
L-Tyrosine	17	7	12	26	14	24
DL-Phenylalanine	7	33	37	34	21	2
L-Cystine	23	26	18	19	11	25
DL-Methionine	0	27	34	39	7	0
DL-Threonine	9	18	19	22	13	15
DL-Serine	37	21	40	34	13	34
DL-Leucine	2	19	30	37	16	0
DL-Isoleucine	43	35	44	43	19	40
DL-Valine	36	30	34	40	28	34
L-Glutamic acid	54	51	29	51	57	48
L-Aspartic acid	29	27	30	30	25	27
Glycine	29	25	28	31	25	26
DL-Alanine	43	20	42	36	32	36
L-Proline	34	19	33	31	28	33
β-Alanine	23	17	22	25	26	22
Adenine·H_2SO_4	30	0	14	10	0	19
Guanine·HCl	33	23	25	18	0	15
Xanthine	25	17	0	0	0	0
L-Asparagine	31	27	33	30	28	36
L-Glutamine	23	23	24	25	23	25
Urea	32	20	32	31	0	0
$(NH_4)_2SO_4$	39	19	42	36	0	0
$NaNO_2$	22	19	27	21	0	0
$NaNO_3$	35	17	12	22	3	0
NH_4Cl	60	44	41	45	17	26
Control nutrient broth	63	59	63	52	43	52

* Figures represent average turbidities (100 − light transmittance) of third and fourth transfers in test media.

oxidation via the Krebs cycle and formation of biacetyl. The formation of biacetyl may represent an accessory system brought into action by the use of a minimal synthetic medium. Such an observation was made by McElroy and Dorfman (1948) who found that pantothenate-deficient cells of *Proteus morganii* produced acetylmethylcarbinol. According to *Bergey's Manual* neither *Proteus morganii* nor *P. fragi* produce acetylmethylcarbinol in complex media.

None of the naturally occurring fatty acids was utilized as a carbon source. This fact indicates that only one of the products of lipolysis, glycerol, can serve as a sole energy source for *P. fragi*. The failure of the fatty acids to support growth may be due to their insolubility or toxicity.

Five polyhydric alcohols—sorbitol, dulcitol, mannitol, adonitol, and glycerol were studied. Only glycerol was a suitable source of carbon. These results are in agreement with the findings of Sebek and Randles (1952).

Members of the normal monohydric alcohol series (methanol through pentanol) were not satisfactory carbon sources. Sodium bicarbonate was not a suitable carbon source.

Nitrogen compounds studied. As is shown in table 3, of the 30 nitrogen compounds studied, 28 were suitable nitrogen sources for at least one of the 6 test strains. These findings indicate that *P. fragi* has a high synthetic ability, being able to utilize inorganic N compounds as well as a wide variety of organic compounds in the absence of exogenous vitamins.

Among the 28 suitable nitrogen compounds were serine, cystine, and aspartic acid. The close relationship between these compounds and compounds involved in the citric acid cycle provides further evidence for the existence of that system in *P. fragi*.

Combined carbon and nitrogen sources. Compounds which served as suitable carbon and nitrogen sources were incorporated into the test media as sole sources of both carbon and nitrogen. Arginine·HCl, histidine·HCl, glutamic acid,

TABLE 4

Utilization of 4 single compounds as sole source of both carbon and nitrogen by 6 strains of Pseudomonas fragi in a salts medium

Compounds Studied	Molarity	Strain					
		$1S_4$	$1H_1$	$1CH_7$	$1CH_6$	$1M_1$	ISC
L-Histidine·HCl	0.031	65*	54	68	60	60	59
L-Arginine·HCl	0.031	50	50	51	53	40	51
L-Glutamic acid	0.039	61	49	60	60	50	55
DL-Alanine	0.076	32	34	25	33	33	39
Control nutrient broth		63	59	63	52	43	52

* Figures represent average turbidities (100 − light transmittance) of third and fourth transfers in test media.

and alanine were tested and found to support growth when supplied as sole source of both carbon and nitrogen. Table 4 indicates the compounds studied, the concentrations used, and the growth response obtained. The concentrations used represent the sum of the concentrations employed in the individual carbon and nitrogen studies.

All 6 test strains utilized the 4 compounds so well that the growth compared favorably to that in nutrient broth. These results indicate a complex amino acid metabolism which warrants more detailed study.

Aroma production. Fruity aroma production was detected in all cultures in which either alanine, leucine, or glutamic acid was the sole carbon source in the inorganic salts medium, or in which threonine was the nitrogen source in the glucose-salts medium. The findings presented here serve to define more closely the early precursors of the fruity aroma component(s), in corroboration of Omelianski's observation (1923).

SUMMARY

Eighteen of 62 carbon compounds studied supported good growth of at least one of 6 test strains of *Pseudomonas fragi*. All of the Krebs cycle intermediates and related compounds tested produced good growth responses. This fact indicates the presence of the Krebs cycle in the test organisms. Glucose, ribose, and gluconolactone were the only carbohydrates utilized. The utilization of ribose and gluconic acid is evidence for the existence of a system of glucose dissimilation alternate to the Embden-Meyerhof-Parnas schema.

Biacetyl was detected in cultures in which fumarate, malate, oxalacetate, or pyruvate was the sole carbon source.

Glycerol was the only polyhydric alcohol which supported good growth of the test strains. Fatty acids, monohydric alcohols, and $NaHCO_3$ were not suitable carbon sources.

Of 30 nitrogen compounds studied, 28 were suitable nitrogen sources. Arginine, histidine, glutamic acid, and alanine supported excellent growth when supplied as sole source of both carbon and nitrogen.

Fruity aroma production was detected in all cultures in which alanine, leucine, or glutamic acid was the carbon source or in which threonine was the nitrogen source.

The data presented indicate that the nutrition of *P. fragi* is similar to that of other pseudomonads in its ability to grow in the absence of exogenous vitamins when an organic carbon compound and an inorganic nitrogen compound are provided.

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