

Nutritional Versatility of a Starch-Utilizing Flavobacterium at Low Substrate Concentrations

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A starch-utilizing yellow-pigmented bacterium, isolated from tap water, was tested for the utilization of 64 natural compounds at a concentration of 1 g/liter by measuring colony growth on agar media. Only 12 carbohydrates and glycerol promoted growth. Growth experiments with the organism in pasteurized tap water supplied with mixtures of substrates at concentrations of 1 or 10 μg of C of each substrate per liter, followed by separate experiments with a number of carbohydrates at 10 μg of C per liter showed that of these 64 natural compounds only sucrose, maltose, raffinose, starch, and glycerol promoted growth at very low concentrations. Also maltotriose, -tetraose, -pentaose, -hexaose, and stachyose, which were not included in the mixtures, enhanced growth, and generation times of 3 to 5 h at 10 μg of C per liter were observed. The organism, which was tentatively identified as a *Flavobacterium* species, thus appeared to be highly specialized in the utilization of glycerol and a number of oligo- and polysaccharides at very low concentrations.

Yellow-pigmented bacteria, usually belonging to the genus *Flavobacterium*, are commonly part of the bacterial flora of waters poor in organic substrates, i.e., distilled water (7), ground water (23), and drinking water (2, 10, 15). Adaptation of these organisms to low concentrations of substrates is indicated by their multiplication in drinking water (1, 12, 14). More specific information has been presented by van der Kooij and Hijnen (20) who isolated a *Flavobacterium* species with very low substrate-saturation constants for starch and glucose (3.9 and 3.3 μg of C per liter, respectively) which predominated in samples of stored slow sand filtrate supplied with starch (10 and 25 μg of C per liter). In additional experiments with starch added in low concentrations to various types of drinking water, yellow-pigmented bacteria also predominated. The nutritional versatility of one of these bacteria was examined to clarify its dependence upon starch or starch-like compounds for growth as well as its taxonomic position. For this purpose, a large variety of naturally occurring amino acids (AA), carboxylic acids (CA), aromatic acids (AR), and carbohydrates and (poly)alcohols (CHA) were tested as sources of carbon and energy for growth, both at high (grams per liter) and very low (micrograms per liter) concentrations.

MATERIALS AND METHODS

Bacterial strain. The organism (strain S12) was isolated from tap water (Municipal Dune Waterworks of

the Hague) prepared from pretreated dune-infiltrated Meuse River water treated by addition of powdered activated carbon, rapid sand filtration, and slow sand filtration. Treated water contained average concentrations of 3.6 mg of dissolved organic carbon per liter and 0.12 mmol of nitrate. After storage at 15°C of 600 ml of this water supplied with 100 μg of starch C per liter, the predominating organism which formed small nonpigmented colonies on Lab Lemco (Oxoid Ltd.) agar (LLA) plates incubated at 25°C was isolated. A similar procedure has been described previously (20).

Media and substrates. LLA contained 5 g of peptone, 3 g of Lab Lemco beef extract, and 12 g of agar (Oxoid no. 3) in 1 liter of demineralized water. The final pH was 7.4. Mineral salts agar (MSA) consisted of 0.5 g of NH_4Cl , 0.5 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, and 12 g of purified agar in 1 liter of demineralized water. The final pH was 6.9.

Substrates tested as sources of carbon and energy for growth at 1 g/liter included 18 AA, 17 CA, 11 AR, and 18 CHA which are listed in Table 1. The compounds were present in sterile solutions of either 1 or 10% (wt/vol). Moreover, with the listed compounds four different mixtures (i.e., the AA mixture, the CHA mixture, the CA mixture, and the AR mixture) were prepared by dissolving equal amounts of carbon of the various substrates in demineralized water. After neutralization with 0.1 N NaOH, the mixtures were autoclaved. For use of individual substrates at low concentrations (micrograms per liter), small volumes of freshly prepared solutions, which were heated at 60°C for 0.5 h, were added to the experimental bottles. Melibiose, melezitose, trehalose, stachyose, maltotriose, maltotetraose, maltopentaose, and maltohexaose were only tested at a low concentration. The latter three compounds were kindly supplied by H. Hokse of

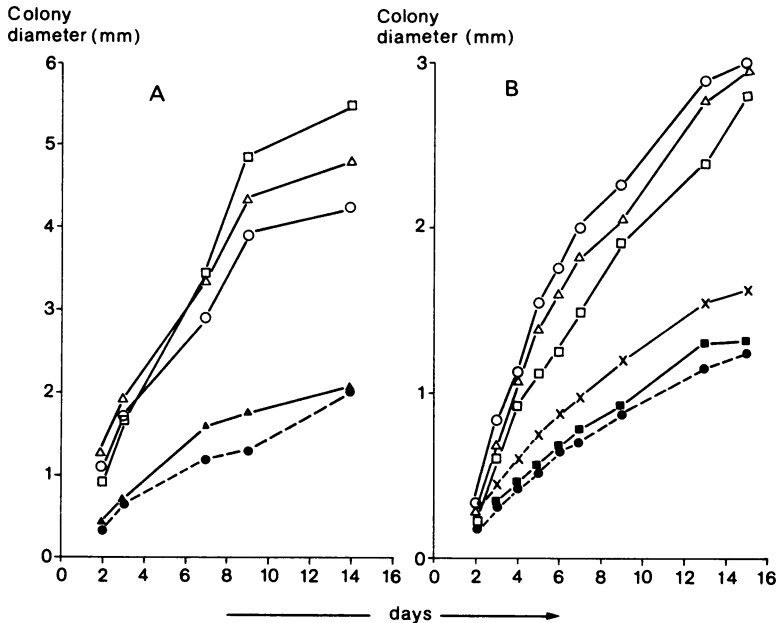


FIG. 1. Increase in diameter of strain S12 colonies during growth at 25°C on LLA (A) and MSA (B), supplied with glucose (○), maltose (△), starch (□), mannitol (▲), succinate (■), or pyruvate (X) at a concentration of 1 g of substrate per liter; ●, shows blanks.

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Growth on agar media. Low numbers (10 to 30) of viable cells grown in tap water supplied with a very low amount of substrate were spread on predried plates (9 cm) of either LLA or MSA with 1 g of substrate per liter. After inoculation, the plates were incubated at 25°C, and during a period of about 2 weeks, diameters of 4 to 5 colonies on each plate (experiments done in duplicate) were measured periodically, using a Wild M7S binocular microscope.

Growth in water. The technique applied to assess the growth of a pure culture in water supplied with various substrates at low concentrations has been described previously (20, 21). The water used for the experiments described in this paper originated from the pumping station Tull and 't Waal (Midden-Nederland Waterworks), where it is prepared from anaerobic ground water by aeration and rapid sand filtration. The final product contained 2.3 mg of dissolved organic carbon per liter and 0.01 mmol of nitrate; the pH was 7.7 (after heat treatment).

Characterization procedures. The following tests were used to characterize the organism: Gram stain, oxidase test (6), oxidation-fermentation test with glucose (5), arginine-deiminase test (18), tests for NO_2^- or N_2 production from NO_3^- (16), and urease activity (17). In addition, the isolate was tested for hydrolysis of proteins (casein, gelatin), starch, chitin, and Tween 80, using a standard agar medium either made turbid with casein or chitin or containing 2.5 g of gelatin, starch, or Tween 80 per liter. All media were incubated at 25°C. The media and procedures for these tests have been described previously (19).

RESULTS

Utilization of substrates present at a concentration of 1 g/liter. Strain S12 multiplied slowly on LLA, and after 48 h of incubation, very small circular convex nonpigmented colonies became visible. Colony size increased upon further incubation, but pigmentation did not appear. With 12 of 18 CHA added to LLA, growth of the colonies was clearly enhanced (Fig. 1A; not all data shown). These colonies rapidly increasing in size, and those on the agars supplied with lactose and inositol, respectively, which did not promote growth, were brightly yellow colored. On MSA, similar growth-favoring effects were observed with CHA (Fig. 1B). The radial growth rates of the colonies on LLA and MSA supplied with CHA had equal maximum values of 8 to 11 $\mu\text{m}/\text{h}$, but the colonies on MSA remained smaller (Fig. 1). The effects on the size of the colonies of all the substrates tested at 1 g/liter are presented in Table 1. From the presented results, it can be concluded that AA, CA, and AR were either not utilized, growth retarding, or lethal when tested at a concentration of 1 g/liter.

Utilization of substrates at low concentrations. As compared with growth of the blanks, growth of strain S12 was clearly enhanced by the addition of the CHA mixture, and by the addition of all mixtures together, i.e., the total mixture (TM), but not by the AA, CA, and AR mixtures

TABLE 1. Effects of 64 different substrates on relative colony size of *Flavobacterium* sp. strain S12 on agar media^a

CHA	R_s^2/R_b^2 (LLA)	R_s^2/R_b^2 (MSA)	AA	R_s^2/R_b^2 (MSA)	CA	R_s^2/R_b^2 (MSA)	AR	R_s^2/R_b^2 (MSA)
L-Arabinose	5.6	5.4	Glycine	0.4	Formate	— ^b	Benzoate	—
D-Xylose	4.5	4.5	L-Alanine	0.6	Acetate	—	<i>p</i> -Hydroxybenzoate	1.1
D-Glucose	4.5	5.3 (6.4) ^c	L-Valine	0.8	Glyoxylate	—	Salicylate	—
D-Mannose	4.2	2.7	DL-Serine	0.4	Oxalate	—	Gallate	—
D-Galactose	4.8	3.3	L-Threonine	0.6	Glycolate	1.1	Anthranilate	—
L-Rhamnose	5.2	2.4	L-Leucine	0.8	Propionate	—	Vanillate	—
Fructose	7.2	3.4	L-Isoleucine	0.7	Pyruvate	1.8	Phthalate	0.7 ^d
Sucrose	7.2	2.6	L-Histidine	0.6	DL-Lactate	—	Ferulate	—
Cellobiose	0.9	0.7	L-Arginine	1.1	Malonate	1.4	Nicotinate	0.5 ^d
Lactose	1.2	1.3 (1.2) ^c	L-Aspartate	1.0	β -OH-butyrate	1.3	<i>p</i> -Hydroxyphenylacetate	0.4 ^d
Maltose	5.8	5.3 (6.1) ^c	L-Asparagine	1.2	Fumarate	1.3	DL-Mandelate	0.5 ^d
Raffinose	5.9	4.0	L-Glutamate	1.3	Succinate	1.1		
Starch	7.6	6.4	L-Glutamine	0.7	Malonate	—		
Gluconate	1.2	1.1	L-Proline	1.0	L-Tartrate	1.5		
Ethanol	1.3	1.7	L-Lysine	0.9	Valerate	NT ^e		
Glycerol	7.8	1.8	L-Tyrosine	1.1	α -Ketoglutarate	1.1		
Mannitol	0.7	1.0	DL-Tryptophan	1.3	Citrate	—		
Inositol	0.9	0.9	DL-Phenylalanine	0.6	Adipate	1.2		

^a Relative colony size is R_s^2/R_b^2 , in which R_s is the radius of a colony grown on agar supplied with a specific substrate and R_b is radius of a colony on agar without this substrate (blank). LLA and MSA are used as blanks. Incubation period is 14 to 15 days.

^b —, No visible colonies developed within 14 days of incubation.

^c Repeated experiment.

^d Number of colonies on the agar supplied with the substrate was about 50% of the number of colonies on the agar without the substrate.

^e NT, Not tested.

TABLE 2. Growth of *Flavobacterium* sp. strain S12 at $15 \pm 0.5^\circ\text{C}$ in tap water supplied with mixtures of naturally occurring substrates in a concentration of 1 μg (A) or 10 μg (B) of C per liter^a

Mixture added	Total concn of added substrate (μg of C/liter) ^b		Generation time (h)		N_{max} (CFU/ml)	
	A	B	A	B	A	B ^c
None (1) ^d			36.7	NG ^e	1.5×10^4	350
None (2) ^d			42.6	NG	1.0×10^4	300
AA (1)	18.1	180	55.6	NG	5.0×10^3	140
AA (2)	18.3	179	46.0	NG	9.1×10^3	160
CA (1)	18.9	180	46.0	NG	1.2×10^4	110
CA (2)	17.1	186	38.3	NG	6.2×10^3	120
AR (1)	10.2	106	48.8	NG	1.5×10^4	100
AR (2)	11.4	111	32.6	NG	1.3×10^4	110
CHA (1)	17.7	178	10.0	3.2	1.1×10^5	8.3×10^5
CHA (2)	18.6	174	10.1	3.4	7.3×10^4	8.8×10^5
TM (1)	63.7	665	11.8	3.5	6.5×10^4	9.1×10^5
TM (2)	68.3	634	10.4	3.5	8.8×10^4	7.4×10^5

^a Starving cells grown at an initial concentration of 100 μg of starch C per liter were used as inoculum (110 to 150 CFU/ml).

^b Total concentration after correction for the water volume deviating from 600 ml.

^c Within an incubation period of 8 days; N_{max} values with CHA and TM were reached within 4 to 6 days.

^d 1 and 2, Duplicate flasks.

^e NG, No growth (no systematic increase of the initial colony counts) within 8 days of incubation.

(Table 2). The maximum colony count (N_{max}) values of the TM and CHA mixture in experiment B were approximately 10 times higher than the N_{max} values of these mixtures in experiment A. Thus, increasing the concentration of substrate did not increase the ability of strain S12 to utilize substrates. Moreover, the similarity of the N_{max} values of the TM and CHA mixture as observed in both experiments indicates that AA, CA, and AR were not assimilated by the cells growing on the CHA.

The components of the CHA mixture which were utilized by strain S12 at a low concentration were identified by separate tests at a concentration of 10 μg of C per liter of those compounds which favored growth at 1 g/liter. Only five substrates, namely, sucrose, maltose, raffinose, starch, and glycerol, were utilized for growth. (Fig. 2; not all negative data shown). The N_{max} of strain S12 obtained for the utilized substrates varied from 1.1×10^5 CFU/ml (maltose) to 2.5×10^5 CFU/ml (sucrose), and the generation times varied from 4.5 h (sucrose) to 12 h (maltose). These observations explain the N_{max} values presented in Table 2. In addition to the sugars included in the CHA mixture, melibiose, melezitose, trehalose, stachyose, maltotriose, maltotetraose, maltopentaose, and maltohexaose were tested at 10 μg of C per liter. Only with the five latter compounds was a rapid growth (generation time varied from 3 to 5 h) observed, with N_{max} values of about 2×10^5 CFU/ml.

Characterization of strain S12. In addition to the substrates which could serve as sources of

carbon and energy for growth of strain S12, several additional properties of this bacterium have been determined (Table 3). The organism was identified as a non-proteolytic *Flavobacterium* species. In mineral salts medium (identical to MSA without agar) containing 100 mg of starch C per liter, the maximum level of growth (measured as extinction at 450 nm) was not influenced by replacement of NH_4^+ with an equivalent amount of NO_3^- . Therefore, both nitrogen compounds may serve as a source of nitrogen, and it is concluded that the organism does not require specific growth factors.

DISCUSSION

Significance of characterization procedures. Procedures to characterize bacteria usually include tests for the production of acids from CHA in peptone media under aerobic and anaerobic conditions. The *Flavobacterium* sp. strain S12 studied in this paper and *Flavobacterium* sp. strain 166 described previously (20) did not produce acids from glucose in the oxidation-fermentation test (5). However, glucose as well as a number of other CHA appeared to be suitable substrates for growth (Table 1), but the radial growth rates of the colonies of strain S12 are very low as compared with those of *Escherichia coli* (8) and *Pseudomonas fluorescens* (13) growing on MSA with glucose. Hence, a technique more sensitive than simple replica plating had to be used to demonstrate the utilization of these and other substrates added to MSA. It is possible that the substrate concentration of 1 g/liter is unfavorable for *Flavobacterium* sp.

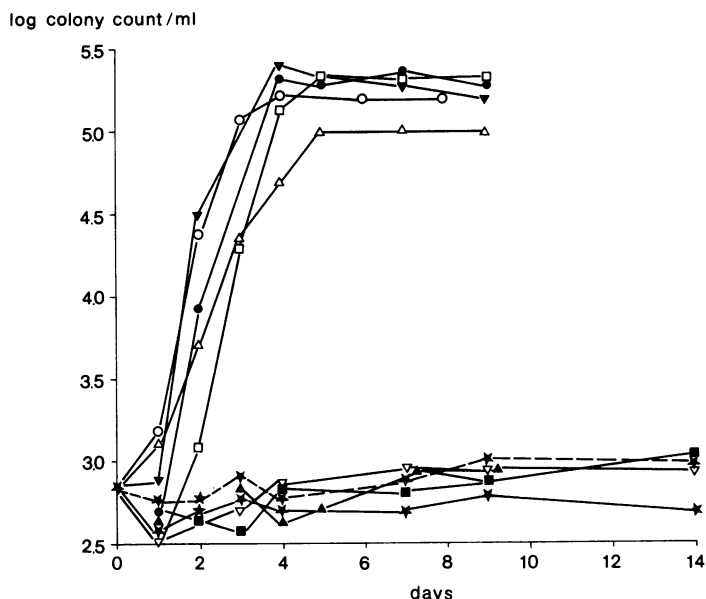


FIG. 2. Growth curves of strain S12 at $15 \pm 0.5^\circ\text{C}$ tap water supplied with glucose (▲), maltose (△), raffinose (▽), starch (○), lactose (■), glycerol (□), sucrose (▼), or rhamnose (▽) at a concentration of $10 \mu\text{g}$ of C per liter. These were all single growth experiments, except those with the blanks (★), which were done in duplicate.

strain S12, which grows rapidly in water at very low concentrations of substrates.

Isolate S12 fits well into the description of the genus *Flavobacterium* as given by Holmes and Owen (4), but none of the species of this genus as described in *Bergey's Manual of Determinative Bacteriology* (22) includes a combination of properties as observed with *Flavobacterium* sp. strain S12, which is amylolytic and non-proteolytic (Table 3). The organism also does not resemble groups of *Flavobacterium*-like bacteria defined by McMeekin et al. (9) or Hayes (3), in which the amylolytic bacteria were also proteolytic. These observations once more indicate the incomplete description of species belonging to the genus *Flavobacterium*. CHA are suitable substrates to differentiate among bacteria belonging to a specific genus (16, 19). Therefore, it is suggested to differentiate among *Flavobacterium* species on the basis of utilization of these compounds and not on acid production. Additional significant information may be obtained from tests on the production of extra cellular enzymes (proteases, amylases, etc.).

The experiments described in this paper reveal that apart from a taxonomic value, tests for growth at high concentrations of CHA and other substrates only have limited ecological significance. Compounds which were not utilized at high concentrations also did not stimulate growth at low concentrations (1 and $10 \mu\text{g}$ of C per liter). However, a number of compounds

which clearly favored growth at 1 g/liter gave negative results at low concentrations. Unfortunately, tests to measure growth with substrates present at low concentrations are time consuming. The results presented in this study demonstrate that this disadvantage may be minimized by combining tests at high concentrations with those with mixtures of substrates at low concentrations.

Growth of strain S12 at very low substrate concentrations. At concentrations equal to or below $10 \mu\text{g}$ of C per liter, *Flavobacterium* sp. strain S12 only utilized a number of oligosaccharides, the polysaccharides included in starch (amylose and amylopectin), and glycerol. The monosaccharides of which these carbohydrates consisted (glucose, galactose, and fructose), as well as arabinose, xylose, mannose, and rhamnose, did not favor growth at these concentrations, although they were utilized at a concentration of 1 g/liter (Table 1). Obviously, the molecules of oligo- and polysaccharides were more efficiently transported into the cells at very low concentrations than those of the monosaccharides. This finding may reveal the specific character of *Flavobacterium* sp. strain S12. *Flavobacterium* sp. strain 166 studied previously (20) was also found to grow more rapidly with starch than with glucose, suggesting that this property is not uncommon amongst the amylolytic species of the genus *Flavobacterium*. Moreover, the isolation of yellow-pigmented

TABLE 3. Some additional properties of *Flavobacterium* sp. strain S12

Property	Isolate characteristic
Shape of cells	Rods (1.5 to 2 by 0.5 μm) ^a
Motility	—
Gram stain	—
Oxidase	+
Acid from glucose:	
Oxidation	—
Fermentation	—
Arginine deiminase	—
Urease	—
NO ₂ ⁻ from NO ₃ ⁻	—
N ₂ from NO ₃ ⁻	—
Casein hydrolysis	—
Gelatin hydrolysis	—
Starch hydrolysis	+
Chitin hydrolysis	—
Tween 80 hydrolysis	—
Growth at 37°C	—

^a Cells grown at 25°C in mineral salts medium (without agar) with 1 g of starch per liter. Cells on LLA had similar dimensions, but after prolonged incubation (>1 week), elongated forms (25 to 40 μm) without clearly visible septa were observed.

bacteria from tap water enriched with very low concentrations of starch suggests that a high affinity for starch is typical for species of bacteria belonging to the genus *Flavobacterium*.

Flavobacterium sp. strain S12 did not grow in the slow sand filtrate from which it had been isolated (data not shown) despite its high affinities for glycerol and a number of carbohydrates. However, *Flavobacterium* sp. strain 166, which was isolated from the same source, multiplied in this water, possibly as result of its ability to utilize CA in addition to carbohydrates (20). Growth of strain S12 was occasionally observed in the tap water prepared from anaerobic groundwater, as is demonstrated by the differences in growth of the blanks with water of the same source collected at different times (Table 2). The described properties of the isolate suggest that if growth occurs, it is the result of the presence of maltose- and starch-like compounds in the water. Generally, such compounds may originate from natural sources, including glyco- gen and polysaccharides derived from bacteria and algae, but they may also have been introduced into the water during treatment. This applies particularly to starch-based compounds which are frequently used as coagulant aids in water treatment processes to prepare drinking water from surface water (11). The presence of small amounts of such compounds in drinking water may promote biological processes in the distribution system. Chemical methods for the assessment of these compounds occurring at low

concentrations (<1 mg/liter) are lacking. For these reasons, growth experiments with strain S12 may be used to obtain information about the presence of maltose- and starch-like compounds in tap water and in water at various treatment stages.

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