Genome Deoxyribonucleic Acid of the Genus *Zymomonas* Kluyver and van Niel 1936: Base Composition, Size, and Similarities

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Some features of deoxyribonucleic acid (DNA) from 41 *Zymomonas* strains from Zaïrese palm wines, Mexican pulque, and English beer and cider were determined. DNA base composition varies within the narrow range of 47.5 to 49.5 mol% guanine plus cytosine. DNA:DNA hybridizations in stringent conditions show high nucleotide sequence similarities of over 76% DNA duplexing (% D), but mostly over 90% D. There is one slightly aberrant strain with less than 32% D. The molecular weight of the *Zymomonas* genome is 1.5×10^9 . *Z. mobilis* and *Z. anaerobia* have almost identical genomes. Our data do not support species differentiation.

For the last few decades, the bacterial genus *Zymomonas* has been rather neglected by taxonomists, in spite of its economic importance for the production of alcoholic beverages in the tropics and for the deterioration of cider and beer. These facultatively anaerobic, lophotrichous rods ferment glucose, fructose, and sometimes sucrose, with the formation of nearly equimolar amounts of ethanol and CO₂. *Zymomonas mobilis* has been isolated from Mexican pulque (10). This strain was studied by Kluyver and Hoppenbrouwers (9). Related strains were reported from *Arenga* palm wines in central Java (13) and from *Elaeis* and *Raphia* palm wines in western (16) and northern Zaïre (16; see Table 1). A similar organism, *Z. anaerobia*, was isolated from English beer (14, 15). Some other isolates have been reported from cider (*Z. anaerobia* subsp. *pomaceae* [the cider-sickness bacillus], reference 12), apple pulp (1), and pale-ale (2). The metabolism and ecology of *Zymomonas* were reviewed by Dadds and Martin (2). Our *Zymomonas* collection contains nearly all known strains; the ones used here are specified in Table 1.

For a sound taxonomy, the base composition percent guanine plus cytosine (G+C), the degree of deoxyribonucleic acid (DNA) relatedness or percent DNA duplexing (% D) (determined by DNA:DNA hybridizations), and the genome size need to be known. They are reported here.

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains used are listed in Table 1. They were maintained in a medium containing 0.5% (wt/vol) yeast extract (Difco) and 2% (wt/vol) glucose in dis-

tilled water. They were transferred monthly to fresh medium. Cells were grown for 24 h at 30 °C in Erlenmeyer flasks containing 700 ml of medium. Each flask was inoculated with 70 ml of a young culture (20 to 24 h).

Preparation of DNA. All DNA samples used for melting temperature (T_m , i.e., midpoint of the absorbance-temperature curve in degrees Celsius) determinations were prepared by the method of Marmur (11). For hybridization experiments very pure DNA is needed. It was prepared by a procedure slightly modified from that of Schilperoort (Ph.D. thesis, University of Leiden, Leiden, The Netherlands, 1969). The following reagents were prepared. SSC buffer (1×) consisted of 150 mM NaCl and 15 mM trisodium citrate (pH 7.0). Concentrated or diluted SSC buffers are represented by, e.g., 3× SSC, 0.1× SSC, etc. Buffer A consisted of 6 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1 mM ethylenediaminetetraacetic acid (EDTA), 179 mM NaCl, and 10 mM tris(hydroxymethyl)aminomethane (Tris) (pH 9.0). Buffer B contained SSC, 1 mM EDTA, and 1 mM Tris (pH 7.8). The phenol mixture consisted of 1 volume of distilled phenol, saturated with 1 volume of 3× SSC, containing 0.1% 8-hydroxyquinoline. The ribonuclease (RNase) solution contained 2 mg of RNase A plus 600 U of RNase T1 per ml of distilled water at pH 5. This solution was boiled for 10 min and preserved at -12 °C. Pronase solution was made by incubating 0.2% Pronase in 1 M NaCl at pH 7 overnight at 37 °C. It was preserved at -12 °C.

One gram of freeze-dried cells was suspended in 30 ml of buffer A at 70 °C and incubated for 15 min. Solid sodium-laurylsulfate (1.6%, wt/vol) and 0.6 volume of Pronase solution were added. The mixture was adjusted to pH 8.0 in a pH meter and incubated for 3 h at 60 °C. After lysis, 6% (wt/vol) of solid sodium *p*-aminosalicylate and 1 volume of phenol mixture were added; the mixture was shaken for 30 min and centrifuged at 10,000 rpm for 10 min. The supernatant was extracted with chloroform and the latter was removed by centrifugation. The superna-

TABLE 1. *Origin of the Zymomonas strains used*

Strain designation	Isolated from	Place of isolation	Year of isolation	Isolated by
<i>Zymomonas</i> Z1, Z2, Z3, Z5, Z6, Z7, Z8	<i>Elaeis</i> sap	Kinshasa, Zaïre	1967	
<i>Zymomonas</i> VP1, VP2, VP3, VP4	<i>Elaeis</i> sap	Kinshasa, Zaïre	1969	J. Swings
<i>Zymomonas</i> 7.4	<i>Elaeis</i> sap	Tshela, Zaïre	1969	J. Swings
<i>Zymomonas</i> 70.1, 70.2, 70.3, 70.7, 70.9, 70.10, 70.11, 70.12, 70.14	<i>Elaeis</i> sap	Kinshasa, Zaïre	1970	J. Swings
<i>Zymomonas</i> 5.1, 5.3, 5.4, 5.5	<i>Raphia</i> sap	Bwamanda, Zaïre	1971	J. Swings
<i>Zymomonas</i> 17.1, 17.2, 17.3, 17.4	<i>Raphia</i> sap	Mobayi, Zaïre	1971	J. Swings
<i>Zymomonas</i> 42.1, 42.2, 42.3, 42.4	<i>Raphia</i> sap	Bwamanda, Zaïre	1971	J. Swings
<i>Z. anaerobia</i> subsp. <i>po-maceae</i> T. H. Delft	Cider	Bristol, England	Before 1951	Deposited by B. T. P. Barker (possibly strain 1 of Millis [12])
<i>Z. anaerobia</i> NCIB 8227	"Bad" beer	England	Before 1951	Almost certainly in the Research Dept., H. J. Bunker, Barclay
<i>Z. anaerobia</i> Queens-land 409 ex NCIB 8227	"Bad" beer	England		Perkins & Co Ltd., London (H. J. Bunker, personal communication)
<i>Z. mobilis</i> Queensland 410 ex NCIB 8938 ex ATCC 10988	Agave sap	Mexico	1925	P. Lindner
<i>Z. mobilis</i> ATCC 10988	Agave sap	Mexico	1925	P. Lindner
<i>Z. anaerobia</i> 1	Beer	England	1970	M. J. S. Dadds
<i>Z. anaerobia</i> 2	Beer	England	1970	M. J. S. Dadds
<i>Z. anaerobia</i> 2.1	Subclone of 2		1972	J. Swings
<i>Z. anaerobia</i> 2.2	Subclone of 2		1972	J. Swings

tant was dialyzed against freshly made buffer B at room temperature. After 1 day, 0.05 volume of RNase solution was mixed in, and dialysis was continued against fresh buffer B for 4 h at 37 C. At that time, 0.6 volume of Pronase solution was added. Dialysis was continued at 37 C first against buffer B in 1 M NaCl at pH 8.0 for 16 h, and then against buffer B at pH 7.8 for 2 days at room temperature. The solution was then made up at 37 C to 1% solid sodium laurylsulfate (dissolved by gentle shaking), 6% solid sodium *p*-aminosalicylate, and 3% NaCl, added in that order. To the clear solution was added 1 volume of phenol mixture; the complete mixture was shaken gently for 1 h at room temperature and centrifuged. The aqueous supernatant was removed and repeatedly deproteinized with chloroform until no protein interphase was visible. Pure DNA, containing only barely detectable traces of protein and ribonucleic acid, was precipitated with ethanol, dissolved in $0.1 \times \text{SSC}$, and dialyzed against $0.1 \times \text{SSC}$ at 4 C.

Determination of DNA base composition. The melting point (T_m) of DNA samples was determined from thermal denaturation curves in SSC buffer, as described by De Ley and Van Muylen (6). The DNA base composition, expressed as G+C content, was calculated from the equation $\text{mol\% G+C} = 2.44 (T_m - 69.4)$ (reference 4).

Genome size. The genome size was determined with the method of the initial renaturation rates (7; Gillis and De Ley, manuscript in preparation).

DNA:DNA hybridizations. For DNA:DNA hybridizations, a variant of the above method was used (5). All hybridizations were carried out in stringent conditions, i.e., $2 \times \text{SSC}$ and 72 C. The results are expressed as the degree of binding or duplexing % D, defined as the percentage of the total DNA from two genomes of the same size which can hybridize and which have the same or very similar nucleotide sequences (5).

RESULTS

DNA base composition. For every strain, at least two DNA denaturation curves were recorded. The T_m values, the mean G+C content, the heterogeneity of base distribution within the genome, expressed as mean σ values, and the percent hyperchromicity are listed in Table 2. All strains have a G+C value within the narrow range of 47.5 to 49.5 mol%. The mean T_m is 89.3 ± 0.2 C (standard deviation), corresponding to a mean G+C content of 48.6 ± 0.6 mol%. Two strains have slightly lower G+C values. Strain 70.11, with 47.5 mol% G+C, is a

TABLE 2. *Zymomonas* DNA base composition^a

<i>Zymomonas</i> strain designation	T_m in $1 \times \text{SSC}$ (°C)	Mean G+C (mol%)	Mean σ (°C)	Hyperchromicity (%)
Z1	89.4 $\pm$ 0	48.9	1.65	28
Z2	89.45 $\pm$ 0.15	49.0	1.83	29
Z3	89.05 $\pm$ 0.25	48.0	1.80	30
Z5	89.15 $\pm$ 0.15	48.3	1.73	28
Z6	89.4 $\pm$ 0	48.9	1.76	23
Z7	89.5 $\pm$ 0	49.1	1.75	25
Z8	89.45 $\pm$ 0.15	49.0	1.73	25
VP1	89.25 $\pm$ 0.15	48.5	1.90	28
VP2	89.15 $\pm$ 0.05	48.3	1.90	25
VP3	89.3 $\pm$ 0.2	48.6	1.77	23
17.1	89.2 $\pm$ 0.1	48.4	1.68	28
17.2	89.15 $\pm$ 0.05	48.3	1.65	23
17.3	89.2 $\pm$ 0.1	48.4	1.93	26
17.4	89.65 $\pm$ 0.15	49.5	1.60	27
42.1	89.5 $\pm$ 0.1	49.2	1.75	26
42.2	89.6 $\pm$ 0.1	49.4	1.75	29
42.3	89.65 $\pm$ 0.25	48.4	1.73	23
42.4	89.4 $\pm$ 0.2	48.9	1.75	24
70.1	89.2 $\pm$ 0	48.4	1.60	24
70.2	89.25 $\pm$ 0.05	48.5	1.63	19
70.3	89.45 $\pm$ 0.15	49.0	1.78	30
70.7	89.05 $\pm$ 0.05	48.0	2.03	30
70.9	89.25 $\pm$ 0.15	48.5	1.75	29
70.10	89.45 $\pm$ 0.05	49.0	1.70	25
70.11	88.85 $\pm$ 0.05	47.5	1.58	27
70.12	89.1 $\pm$ 0.1	48.2	1.48	28
70.14	89.1 $\pm$ 0.1	48.2	1.70	28
5.1	89.45 $\pm$ 0.05	49.0	1.85	30
5.3	89.6 $\pm$ 0	49.4	1.78	24
5.4	89.35 $\pm$ 0.05	48.8	1.85	30
5.5	89.05 $\pm$ 0.25	48.0	1.63	27
7.4	89.3 $\pm$ 0	48.6	1.70	27
1	89.35 $\pm$ 0.25	48.8	1.50	28
2.1	89.2 $\pm$ 0.1	48.4	1.70	28
ATCC 10988	89.5 $\pm$ 0	49.1	1.73	20
410	89.3 $\pm$ 0	48.6	1.65	28
NCIB 8227	89.45 $\pm$ 0.15	49.0	1.88	25
subsp. <i>pomaceae</i>	88.9 $\pm$ 0	47.7	1.68	19

^a All T_m measurements were carried out in duplicate. The mean G+C content was calculated with the equation of De Ley (4).

quite normal representative of the Zaïrese *Zymomonas*, as shown by both genotypic (see below) and phenotypic (Swings and De Ley, manuscript in preparation) measurements. *Z. anaerobia* subsp. *pomaceae* with 47.7 mol% G+C is a separate case; this strain has abnormal genotypic (see below) and phenotypic (Swings and De Ley, manuscript in preparation) features. We repeatedly tried to prepare DNA from strain VP4 without success.

A few G+C values have been published; they fit well in the G+C range for *Zymomonas* which we describe here. Dadds et al. (3) found 48.3 mol% G+C for *Z. mobilis* NCIB 8938. Kiehn and Pacha (8) reported 48.6 mol% G+C, presumably for the same strain. For strain

ATCC 10988 we found 49.1 mol% G+C, and for its subculture 410, we found 48.6 mol% G+C. Dadds et al. (3) reported 47.6 mol% G+C for *Z. anaerobia* NCIB 8227; we found 49.0 mol% G+C. They also reported 46.4 mol% G+C for *Z. anaerobia* NCIB 10565 and 45.1 mol% G+C for *Z. mobilis* B70. Neither strain was available to us.

Genome-DNA relatedness. To determine the degree of DNA duplexing % D between every pair of strains from our collection would be very laborious and time consuming. Practice shows that it is not necessary. Therefore, we hybridized first the DNA of every strain with the DNA of reference strain 5.3. This strain was chosen because it is phenotypically very

representative and because of its good DNA yield. The DNA relatedness of all strains, except one (*Z. anaerobia* subsp. *pomaceae*), is at least 76% D with strain 5.3 (Table 3). The hybridization conditions were very stringent ($2\times$ SSC, 72 C, or 21 C below $T_{m, 2\times SSC}$), i.e., those which allow duplex formation to occur only between very closely related or identical polynucleotide sequences. We determined also the degree of duplexing between several other pairs of DNA (Fig. 1). The average DNA relatedness of the Zaïrese strains is $94 \pm 6\%$ D. Twenty-eight strains are very similar with at least 88% DNA duplexing. The relatedness of three other Zaïrese strains is somewhat lower—17.2 (84% D), 70.10 (85% D), and 70.12 (86% D). There appears to be a second group of very high genome-DNA relatedness, consisting

TABLE 3. Degree of DNA duplexing (% D) of each strain with strain 5.3^a

Strain designation	D $\pm$ s (%)
Z1	94 $\pm$ 2.5
Z2	101 $\pm$ 8
Z3	91 $\pm$ 8
Z5	96 $\pm$ 4
Z6	96 $\pm$ 3.5
Z7	93 $\pm$ 3
Z8	96 $\pm$ 5
7.4	96 $\pm$ 3.5
17.1	89 $\pm$ 3
17.2	84 $\pm$ 6
17.3	98 $\pm$ 6
17.4	95 $\pm$ 2
VP1	96 $\pm$ 5
VP2	96 $\pm$ 6
VP3	97 $\pm$ 4
42.1	97 $\pm$ 6
42.2	99 $\pm$ 10
42.3	94 $\pm$ 0
42.4	98 $\pm$ 4
70.1	92 $\pm$ 1
70.2	97 $\pm$ 1
70.3	97 $\pm$ 3
70.7	95 $\pm$ 9
70.9	93 $\pm$ 5
70.10	85 $\pm$ 4
70.11	93 $\pm$ 7
70.12	86 $\pm$ 1
70.14	88 $\pm$ 3
5.1	98 $\pm$ 4
5.4	98 $\pm$ 2
5.5	98 $\pm$ 6
2.1	84 $\pm$ 8
2.2	78 $\pm$ 1
NCIB 8227	84 $\pm$ 4
ATCC 10988	76 $\pm$ 3
subsp. <i>pomaceae</i>	32 $\pm$ 4

^a In $2\times$ SSC at 72 C, determined with the initial renaturation rate method (5).

FIG. 1. Genome DNA similarities in *Zymomonas*, determined by DNA:DNA cross-hybridization. The data are expressed in percent DNA duplexing. In the cases marked with an asterisk, only one or two hybridizations could be carried out because of the small amount of DNA available.

of *Z. mobilis* ATCC 10988 and Queensland 410, *Z. anaerobia* NCIB 8227 and Queensland 409, *Z. anaerobia* 2.2 and 2.1 and the Zaïrese *Zymomonas* strains 17.2, 70.12, and 70.10. The average % D within this group is 96; its average % D with the 5.3 group is 82. The strain *Z. anaerobia* subsp. *pomaceae* is distinctly different, having less than 32% D with all other strains.

Genome sizes. We compared the renaturation rates of our DNA samples among each other and to a reference strain of *Escherichia coli* B with a known genome molecular weight (M) of 2.71×10^9 and a known apparent renaturation rate constant k' of 0.222. The mean k' value from 99 determinations of the renaturation rate of *Zymomonas* 5.3 DNA is 0.396 ± 0.050 . We applied an equation from Gillis et al. (7): $k'_{5.3} \times M_{5.3} = k'_{coli} \times M_{coli}$. The molecular weight $M_{5.3}$ of the genome DNA of *Zymomonas* strain 5.3 is $(1.53 \pm 0.19) \times 10^9$. The k' values from the other *Zymomonas* strains do not differ significantly from $k'_{5.3}$.

DISCUSSION

All strains of our *Zymomonas* collection, except *Z. anaerobia* subsp. *pomaceae*, have very similar genome DNA. The DNA base composition is almost the same at 48.5 ± 1.0 mol% G+C. The base sequence similarity in stringent hybridization conditions is very high and points to almost complete identity of many strains. The Zaïrese strains were isolated at quite different times and in widely different locations. Their DNA hybridizes very high with DNA from the British and the Mexican strains of a quite different habitat. This might indicate either very little recent evolutionary diver-

gency in the genus *Zymomonas* or a rather recent dispersion of this genus over the continents. It is interesting to note the almost complete genetic identity between *Z. mobilis* and *Z. anaerobia*. A species differentiation is thus not supported. Dadds et al. (2, 3) reached the same conclusion on different grounds.

The strain of *Z. anaerobia* subsp. *pomaceae* is genetically rather different from all our other strains. Its genome relatedness is less than 32% D with all other *Zymomonas* strains studied here. We shall show later that this is reflected in its phenotypic behavior. We shall report on the taxonomic and nomenclatural implications more closely in the near future.

The genome size of *Zymomonas* is rather small: 56% of the *Escherichia coli* genome. It can be estimated that it can accommodate some 1,500 cistrons. There are quite a number of bacterial genera with genomes that small (7).

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REPRINT REQUESTS

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