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Aggregation of 27 oral bacteria by human whole saliva.
Influence of culture medium, calcium, and bacterial cell concentration, and interference by autoaggregation

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Abstract. Twenty-seven oral strains of the genera *Actinomyces* (5), *Bacteroides* (3), and *Streptococcus* (19) were tested for aggregation by human whole saliva, as well as the effect of culture medium, Ca-ions, and bacteria concentration thereupon. Of the media tested, GF-broth gave rise to less interference by autoaggregation or higher aggregation titers than BHI and TSB, and was used throughout this study. In most cases, Ca-ions (1 mM) only enhanced the rate of induced aggregation, whereas raising the bacteria concentration increased the rate of both induced- and autoaggregation. The final titers, ranging from 1–64, were hardly affected by these parameters, except those of *S. rattus* HG 59 and *S. mutans* HG 199, which were respectively increased and decreased by Ca-ions. Saliva-induced aggregation was observed for 21 strains of *A. viscosus, A. naeslundii, A. israelii, B. gingivalis, B. intermedius, S. cricetus, S. mutans, S. rattus, S. sanguis,* and *S. sobrinus,* mostly within 15 min to 3 h. Seventeen of these strains also showed autoaggregation, usually well after the onset of induced aggregation. Any potential induced aggregation of *B. gingivalis* HG 91 was always masked by autoaggregation, as well as that of the *S. mutans* strains under a particular set of conditions. The aggregation rate and titer varied considerably in a mutually unrelated and strain-dependent way. These microtiterplate data were matched by the 5 spectrophotometric patterns observed for saliva-bacterial interaction, which moreover, gave the better differentiation between induced and autoaggregation. In conclusion, most strains tested can show rapid saliva-induced aggregation in a strain-dependent way, yet strongly affected by the experimental conditions and interference from autoaggregation.

Introduction

The human oral cavity harbors a variety of bacterial species, both free in saliva and adhered to oral surfaces, of which a number is involved in oral diseases. Infections start with bacterial adherence to oral surfaces, nonadhering bacteria being washed away and discarded by the continuous flow of saliva. Upon adherence, bacteria must be able to multiply at a sufficient rate to overcome

the cleansing effect of desquamation, salivary flow and mechanical forces. On teeth, the resulting bacterial masses are known as dental plaque, considered to be the cause of dental caries and periodontal diseases (Gibbons & Van Houte 1975).

Various factors interfere with bacterial adherence (Abraham & Beachey 1985), of which a number by interaction with bacterial surface components, e.g., adhesin specific sIgA (McNabb & Tomasi 1981; Hanson et al. 1983), and host-receptor analogues (Gibbons 1982). Thus, the adherence of several oral bacteria is inhibited by preincubation with saliva, presumably by blocking or steric hindrance of bacterial adhesins (Ørstavik et al. 1974; Gibbons 1982). These interactions often result in bacterial aggregation as demonstrated for certain strains of *Streptococcus* (Ørstavik et al. 1974) and *Actinomyces* (Ellen et al. 1983). According to microscopic observations on the colonization of dental surfaces (Lie 1977), aggregates are not primarily involved in adherence. Bacterial aggregation is therefore considered to promote bacterial clearance from the oral cavity (Mandel 1979; Gibbons 1982).

For this reason we studied the aggregation-inducing ability of human whole saliva towards 27 oral strains of known pathogenetic species, as well as the influence of Ca-ions, culture medium and bacterial cell concentration thereupon in a microtiterplate assay. For comparison, the strains were also tested under one set of experimental conditions in a spectrophotometric assay according to Ericson et al. (1975).

Materials and methods

Bacteria

The organisms used in this study are listed in Table 1. Strains, previously described as *Streptococcus mutans,* are renamed as *S. cricetus, S. rattus,* and *S. sobrinus* as proposed by Coykendall (1977). Own isolates were characterized as *S. mutans* and *S. sanguis* I & II according to Facklam (1977). Strains were maintained, cultured, and prepared as described previously (Koop et al. 1988). Besides growth in a trypticase peptone broth (BBL) according to Gibbons & Fitzgerald (1969), hence GF-broth, 8 streptococcal strains, frequently encountered in literature, were cultured for comparison in brain heart infusion, BHI (Difco), and in trypticase soy broth, TSB (BBL). Based on aggregation data of these strains in microtiterplates (Table 2), GF-broth was selected for the culturing of *Actinomyces* and *Streptococcus* henceforth. *Bacteroides* was grown in BOB's medium according to Shah et al. (1976). Bacterial suspensions in potassium phosphate buffer, KPB (10 mM, pH 7), were standardized to an optical density of 1.5 ± 0.02 at 700 nm (Ericson et al. 1975).

Table 1. Strains used in this study.

Species		Strain	Source*
Streptococcus sanguis I		HG 169	1
		HG 295 (NCTC 7863)	2
		HG 311	1
Streptococcus sanguis II		HG 168	1
		HG 179	1
		HG 310	1
Streptococcus mutans	serotype c	HG 207 (C67-1)	3
		HG 217 (ATCC 25175)	4
		HG 455 (MT 8148)	5
	serotype e	HG 58(LM-7)	6
		HG 199	1
		HG 457 (MT 703 R)	5
	serotype f	HG 458 (OMZ 175)	5
Streptococcus sobrinus	serotype d	HG 456 (B 13)	5
	serotype g	HG 459 (6715-DP)	5
Streptococcus cricetus	serotype a	HG 48 (E 49 N)	7
		HG 216 (ATCC 19642)	4
Streptococcus rattus	serotype b	HG 59 (BHT)	6
		HG 454 (FA-1)	5
Actinomyces viscosus		HG 85 (T14-V)	8
		HG 380 (T14-AV)	8
Actinomyces naeslundii		HG 401 (ATCC 12104)	9
		HG 402 (398 A)	9
Actinomyces israelii		HG 345 (ATCC 10048)	10
Bacteroides intermedius		HG 65 (T 588)	11
Bacteroides gingivalis		HG 66 (W 83)	11
		HG 91 (381)	8

* Sources: (1) J. de Graaff; own isolate from dental plaque. (2) Public Health Laboratory Service, UK. (3) J.D. de Stoppelaar. (4) American Type Culture Collection, USA. (5) S. Hamada. (6) D.B. Clewell. (7) Forsyth Dental Center, USA. (8) S.S. Socransky. (9) J.S. van der Hoeven. (10) R.P. Ellen. (11) H.N. Shah.

Saliva

Unstimulated clarified human whole saliva (CHWS) was obtained as reported (Koop et al. 1988), and stored at −20° C after dialysis against KPB with or without 1 mM $CaCl_2$. Prior to application, CHWS was thawed, gently homogenized in a Potter-Elvehjem type homogenizer, and centrifuged (15 min, 2000 × g, 4° C). The protein content was determined according to Lowry et al. (1951). Total calcium in undialyzed CHWS was assessed in a Varian Techtron model AA-100 atomic absorption spectrophotometer.

Aggregation assays

Aggregation was studied both in microtiterplate- and spectrophotometric assay as described previously (Koop et al. 1988). Twofold serial dilutions of CHWS in KPB (100 µl) were mixed with aliquots of bacterial suspension, adjusted with KPB to a total volume of 300 µl, and incubated during 6 h at 37° C. Microtiterplates were checked once more after standing overnight at room temperature. Controls without CHWS were included.

The influence of Ca-ions and bacterial cell concentration was tested in microtiterplate assay. For 16 strains, aliquots of 100 µl of bacterial suspension were tested with and without Ca-ions (1 mM final concentration). In the presence of Ca-ions, both 100 and 200 µl of bacterial suspension were tested for 17 strains. More specifically, 5 different cell concentrations (50–250 µl of bacterial suspension in steps of 50 µl per 300 µl aggregation mixture) were examined for *S. rattus* HG 59. Finally, all 27 strains were tested as 200 µl suspensions in the presence of Ca-ions in both microtiterplate and spectrophotometric assay.

The aggregation titer was defined as the reciprocal of the highest CHWS-dilution capable of inducing aggregation at a certain time. A titer of 1 corresponds to 100 µl of undiluted CHWS, equivalent to approximately 125 µg of protein, per 300 µl of aggregation mixture.

Results

General

Aggregation was usually revealed in microtiterplates as sedimented aggregates, and spectrophotometrically as a three-phase decline in absorbance. CHWS-induced aggregation was observed with a total of 21 strains, for 19 of which within 15 min to 3 h. Figure 1 illustrates the generally observed differences in rate and titer of aggregation, as well as the lack of correlation between them, for 8 of the most strongly aggregated strains. Spontaneous aggregation of the control ("autoaggregation") was observed with 23 strains, for 17 of which well after the onset of induced aggregation. At simultaneous appearance, induced aggregation could usually be distinguished by microtiterplate morphology or spectrophotometric pattern (Fig. 3d, e). For *B. gingivalis* HG 91, however, and for all *S. mutans* strains tested under particular conditions, any potential induced aggregation was fully masked by autoaggregation (Table 3).

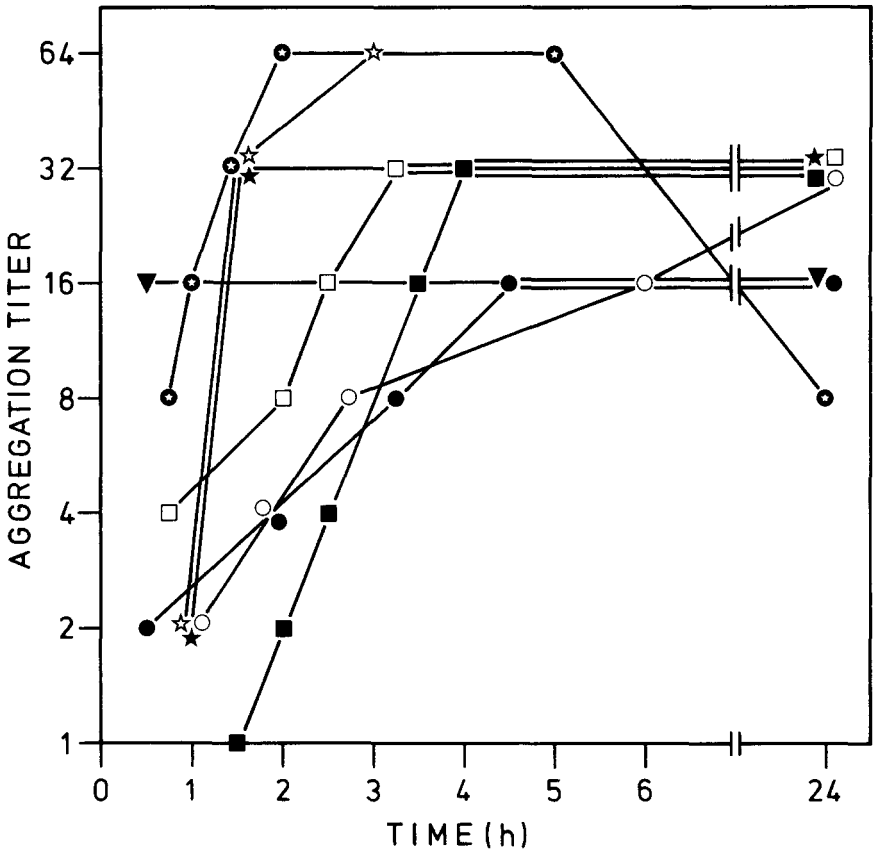


Fig. 1. Aggregation titers at various incubation periods as determined in microtiterplate assay for oral strains, strongly aggregated by CHWS in the presence of Ca-ions (1 mM). Bacterial cell concentration: 100 µl of standardized cell suspension per 300 µl of aggregation mixture. (⊙) *A. israelii* HG 345. (☆) *A. viscosus* HG 85. (★) *A. viscosus* HG 380. (□) *S. mutans* HG 217. (○) *S. rattus* HG 59. (■) *S. sanguis* I HG 295.(▼) *S. sanguis* I HG 311. (●) *S. sanguis* II HG 168.

Microtiterplate assay

Culture medium

Table 2 shows that for the two *S. rattus* and *S. sobrinus* strains the highest and lowest aggregation titers were found after culturing in respectively GF-broth and BHI. Moreover, for *S. sobrinus* the onset of aggregation was advanced in case of GF-broth, whereas interference by autoaggregation was observed in case of TSB. The mere autoaggregation of *S. sanguis* and *S. mutans* was more or less postponed in case of GF-broth. Consequently, GF-broth was used throughout this study.

Table 2. Influence of the culture medium on the onset and titer of saliva-induced aggregation in microtiterplate assay[a].

Species	Strain	Culture medium tested					
		BHI		TSB		GF-broth	
		Onset[b] (h)	Titer[c]	Onset[b] (h)	Titer[c]	Onset[b] (h)	Titer[c]
S. mutans	HG 58	(<0.5)	auto	(<0.5)	auto	(0.5)	auto
S. mutans	HG 207	(<0.5)	auto	(0.25)	auto	(0.5)	auto
S. mutans	HG 458	(0.25)	auto	(0.25)	auto	(1.25)	auto
S. sanguis	HG 295	(0.25)	auto	(0.25)	auto	(0.5)	auto
S. rattus	HG 59	0.5	8	0.5	16	0.5	32
S. rattus	HG 454	0.75	4	0.5	16	0.75	32
S. sobrinus	HG 456	>2.5	2	2.5	± 16	1.25	32
S. sobrinus	HG 459	>6	2	(2.5)	auto	>4	16

[a] Bacterial cell concentration: 200 µl of standard suspension (OD_{700}: 1.5 ± 0.02) per 300 µl of incubation mixture.

[b] Onset of autoaggregation given in parenthesis.

[c] Maximum aggregation titer observed in 24 h of incubation, given as ± when partly, or as "auto" when fully masked by autoaggregation.

Ca-ions

Table 3 shows that particularly the rate of induced aggregation, reflected by the onset, was increased considerably by the presence of Ca-ions for 13 out of 16 strains, whereas the final aggregation titer was hardly affected. Only in case of *S. rattus* HG 59, and to a lesser extent for *B. intermedius* HG 65, Ca-ions also enhanced the aggregation titer, whereas the opposite applied for *S. mutans* HG 199. Except for *A. viscosus* HG 85, the rate of autoaggregation was not enhanced by Ca-ions (not shown). The manifestation of both induced- and autoaggregation was also influenced, being more pronounced in the presence of Ca-ions (not shown).

Bacterial cell concentration

Twice the bacterial cell concentration increased the rate, reflected by the onset, of induced aggregation of at least 10 out of 17 strains (Table 3). As both the rate and extent of autoaggregation were also enhanced, however, the onset and final titer of induced aggregation of a number of strains were masked. For the remaining strains no pronounced effect on the final titer was observed (Table 3), as illustrated in more detail for 5 concentrations of *S. rattus* HG 59 (Fig. 2).

Table 3. Influence of Ca-ions and bacterial cell concentration on the onset and titer of saliva-induced aggregation in microtiterplate assay.

Strain		Onset (h)[a]			Titer[b]					
					within 6 h			at 24 h		
		Bacterial cell concentration (μl/total 300 μl)								
		100		200	100		200	100		200
		Ca-ions								
		−	+	+	−	+	+	−	+	+
S. sanguis I	HG 169	>3	1	0.5	2	2	16	8	16	16
	HG 295	1.5	1.5	(0.5)	32	32	auto	32	32	auto
	HG 311	>3	0.5	1.25	2	16	16	8	16	16
S. sanguis II	HG 168	1.5	0.5	0.5	16	16	16	16	16	auto
	HG 179	>5	4.5	2	–	1	16	8	8	16
	HG 310	>3	1	(0.25)	2	2	auto	4	4	auto
S. mutans	HG 207			(0.5)			auto			auto
	HG 217	1.5	0.75	(0.25)	16	32	auto	16	32	auto
	HG 455			(0.75)			auto			auto
	HG 58			(0.5)			auto			auto
	HG 199	(2)	0.75	(0.25)	auto	2	auto	16	4	auto
	HG 457			(0.25)			auto			auto
	HG 458			(1.25)			auto			auto
S. sobrinus	HG 456			1.25			8			32
	HG 459			>4			16			16
S. cricetus	HG 48			3			64			auto
	HG 216			2.5			16			16
S. rattus	HG 59	>6	1	0.5	–	16	32	2	32	32
	HG 454			0.75			32			32
A. viscosus	HG 85	1.25	1	0.5	64	64	≥ 16	64	auto	≥ 16
	HG 380	1.25	1	0.5	32	32	≥ 16	32	32	32
A. naeslundii	HG 401	(5.5)	1	0.5	auto	2	64	≥ 16	≥ 16	auto
	HG 402	(3.25)	1	0.25	auto	8	64	≥ 8	≥ 8	auto
A. israelii	HG 345		0.75	(0.25)		64	64		≥ 8	64
B. intermedius	HG 65	>6	3	2.5	–	2	16	2	8	16
B. gingivalis	HG 66	>6	>6	>6	–	–	–	1	2	2
	HG 91	(0.5)	(0.5)	(0.25)	auto	auto	auto	auto	auto	auto

[a] Onset given in parenthesis when accompanied and masked by autoaggregation; open space: not tested.
[b] Open space: not tested; –: no aggregation observed; ≥ or "auto": partly or fully masked by autoaggregation.

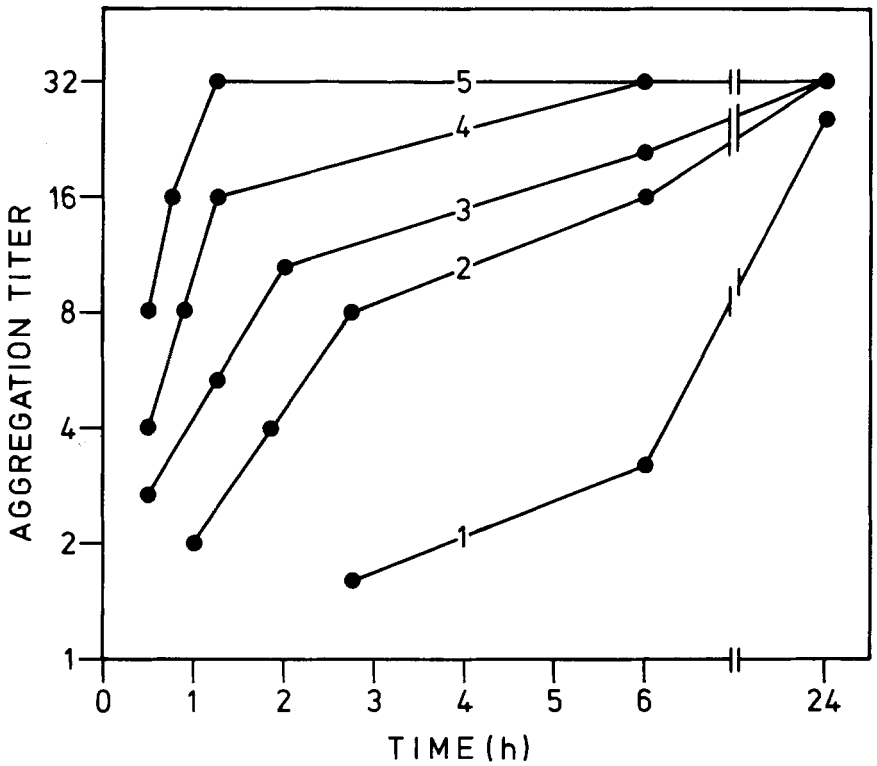
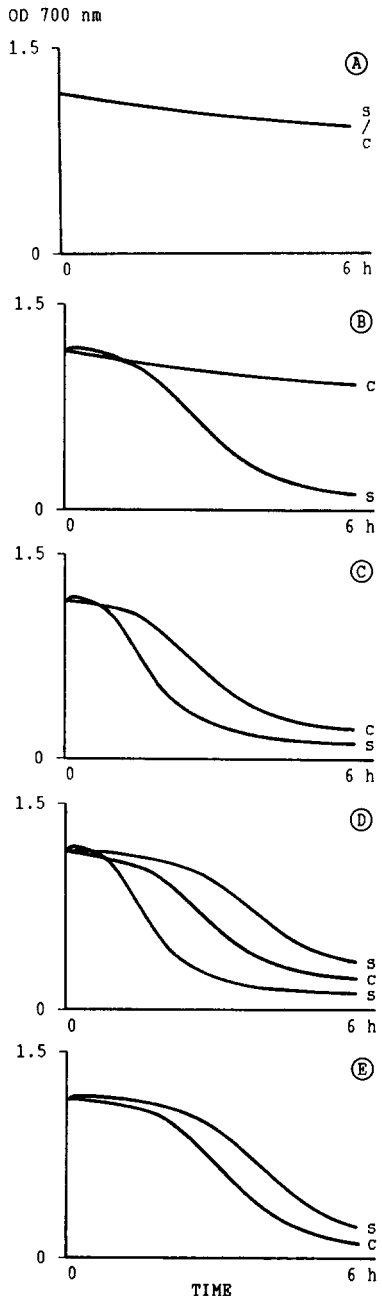


Fig. 2. Influence of the bacterial cell concentration on the rate and titer of CHWS-induced aggregation of *S. rattus* HG 59 in microtiterplate assay. Bacterial cell concentration expressed in μl of standardized suspension per 300 μl of aggregation mixture. (1) 50 μl. (2) 100 μl. (3) 150 μl. (4) 200 μl. (5) 250 μl.

Spectrophotometric assay

In general, the spectrophotometric data matched the microtiterplate results. Five spectrophotometric patterns could be distinguished, as illustrated in Fig. 3a–e with the relevant strains. Only a gradual decrease in absorbance of both the control and CHWS-bacterial mixtures was seen for 1 strain, reflecting the absence of aggregation (Fig. 3a). A marked drop in absorbance just with CHWS was recorded for 8 strains, indicative of induced aggregation without autoaggregation (Fig. 3b). In microtiterplates, however, both *A. viscosus* strains showed nonsedimenting autoaggregates, which are not recorded as such spectrophotometrically. All subsequent patterns show autoaggregation. For 3 strains, the drop in absorbance with CHWS was followed by one in the control, reflecting induced aggregation prior to autoaggregation (Fig. 3c). The



B. gingivalis HG 66

A. viscosus HG 85, HG 380
S. cricetus HG 216
S. rattus HG 59, HG 454
S. sanguis II HG 179
S. sobrinus HG 456, HG 459

S. cricetus HG 48
S. sanguis I HG 169, HG 311

A. israelii HG 345
A. naeslundii HG 401, HG 402
B. intermedius HG 65
S. mutans HG 199
S. sanguis I HG 295
S. sanguis II HG 168

B. gingivalis HG 91
S. mutans HG 58, HG 207, HG 217, HG 455, HG 457, HG 458,
S. sanguis II HG 310

Fig. 3. Schematic representation of the five different spectrophotometric patterns recorded for the interaction of 27 different oral bacterial strains with CHWS (curve S), and of the control bacterial suspension (curve C). (A) Cell sedimentation. (B) CHWS-induced aggregation. (C) CHWS-induced aggregation prior to autoaggregation. (D) CHWS-induced aggregation (titer range approx. 8–64) prior to autoaggregation, followed by delayed aggregation (titer range approx. 1–8). (E) Autoaggregation followed by delayed aggregation (titer range approx. 1–8).

autoaggregation of 7 strains was preceded by induced aggregation with the higher CHWS-dilutions, and followed by delayed aggregation with the lowest CHWS-dilutions (Fig. 3d). Whereas in microtiterplates the induced aggregation of *S. sanguis* I HG 295 and *S. mutans* HG 199 was obscured by autoaggregation (Table 3), it was clearly recorded spectrophotometrically (Fig. 3d). Finally, the remaining 8 strains showed autoaggregation followed by delayed aggregation in the presence of the lowest CHWS-dilutions (Fig. 3e).

Discussion

This study shows that incubation periods of 5 min up to $1^1/_2$ h (McBride & Gisslow 1977; Morris & McBride 1983; Ellen et al. 1983) may be too short to observe the onset or final titer of induced aggregation (Figs. 1–2), whereas periods of 6 to 24 h (Levine et al. 1978) may lead to increased interference by autoaggregation (Table 3). Usually the induced aggregation was largely completed within 6 h, which is therefore concluded to be an appropriate observation period for aggregation in the spectrophotometric and microtiterplate assay.

As bacterial cell surface components are directly involved in adherence and aggregation (Hogg & Embery 1982; Abaas & Holme 1983b; Gibbons et al., Rosan et al., McBride et al. 1985; Clark et al. 1986; Rundegren & Olsson, Van Der Mei et al., Weerkamp et al. 1987), these processes may be influenced by factors affecting the cell surface composition or characteristics (Miller et al. 1978; Abaas & Holme 1982; Rosan et al. 1982; Knox & Wicken 1985; Weerkamp & Handley 1986). Thus, bacterial aggregation is profoundly affected by various nutritional factors (Calleja 1984). In agreement with this are the present data on 3 culture media (Table 2), which mainly differ in pH, and content of protein, total nitrogen, carbohydrate, sodium, potassium and inorganic phosphorus.

Saliva-induced aggregation of oral bacteria can be promoted by Ca-ions in the range of 0.1–0.95 mM (Kashket & Donaldson 1972; Rundegren & Ericson 1981). As the salivary Ca concentration is about 1 mM (Rundegren & Ericson 1981), ranging from 0.75 mM (Kashket & Donaldson 1972) to 1.54 mM (this study) in resting saliva, and 1.1 mM on the average in stimulated saliva (Grøn 1973), we examined the effect of 1 mM $CaCl_2$. In general, a marked increase of the induced-aggregation rate was observed, and just a temporary effect on the titer, except for *S. rattus* HG 59 and *S. mutans* HG 199, whose distinct final titers suggest the presence of a true Ca-activated, respectively inactivated aggregation factor (Table 3). In contrast to results with *S. mitis* (Abaas & Holme 1983a), no marked effect of $CaCl_2$ on autoaggregation was observed in this study. Although yet to be clarified, stimulation by Ca may be caused by

bridging of negatively charged groups on bacteria and agglutinins (Rölla et al. 1979), Z-potential reduction, enabling cells to join (Rundegren & Ericson 1981), stabilization of the agglutinin configuration (Rundegren 1986), or promotion of sIgA-agglutinin interaction (Rundegren & Arnold 1987),

Raising the bacteria concentration generally increased the rate of induced aggregation (Table 3, Fig. 2), although to a lesser extent than that of autoaggregation, which thereby masked potential induced aggregation for a number of strains (Table 3). Instead, most of these strains showed delayed aggregation with the highest CHWS-concentrations (Fig. 3e). As this appeared to be a strain-specific phenomenon, sometimes also observed with induced aggregation (Fig. 3d), it is probably not caused by the viscosity of CHWS-samples, but instead by a rapid coating of bacteria with salivary components, counteracting either induced or autoaggregation. In contrast to the rate of induced aggregation, the final titer, when not masked by autoaggregation, was hardly affected by raising the bacteria concentration, as it remained the same or increased by one step only, suggesting that the bacteria were usually present in excess.

The choice of assay may also affect the manifestation of aggregation (Koop et al. 1988). Nonsedimenting aggregates for instance, observed in microtiterplates, are not detected spectrophotometrically (Fig. 3b, curve C, *A. viscosus* HG 85 & HG 380), whereas minor differences in rate of induced and

Table 4. Influence of experimental conditions on human whole saliva-induced aggregation of five oral bacterial strains at pH 7.2 ± 0.3.

Strain	Experimental conditions[a]							
	Reference no:	[1]	[2]	[3]	[4]	Present study		
	Cult. broth:	BHI	TSB	TSB	BHI	BHI	TSB	GFB
	Incub. time:	2 h	½ h	5 min	5 min	15 min to 6 h		
	Assay:	S	M	M	R	Microtiterplate		
	Temp:	25° C	rt	rt	37° C	37° C		
S. mutans	LM-7 (HG 58)	−		−	+	auto	auto	auto
S. rattus	BHT (HG 59)				±	+	+	+
S. sobrinus	6715-DP (HG 459)				−	−	auto	+
S. sanguis	NCTC 7863[b] (HG 295)	+				auto	auto	+
S. sanguis	ATCC 10556		−	−	+			

[a] References: [1] Hogg SD & Embery G (1979) Archs. Oral Biol. 24: 791–797.
[2] McBride BC & Gisslow MT (1977) Infect. Immun. 18: 35–40.
[3] Morris EJ & McBride BC (1983) Infect. Immun. 42: 1073–1080.
[4] Rosan B, Malamud D, Appelbaum B & Golub E (1982) Infect. Immun. 35: 86–90.
[a] Assays: S – Spectrophotometry; M – Macroscopic observation; R – Radiolabeling of bacteria.
[a] Temp: rt – room temperature.
[b] According to Hogg & Embery (1982) corresponding with *S. sanguis* ATCC 10556.

autoaggregation, unnoticed in microtiterplates, are only revealed spectrophotometrically (Table 3 and Fig. 3d, *A. israelii* HG 345, *S. mutans* HG 199, and *S. sanguis* HG 295).

The influence of experimental conditions on the outcome of aggregation is also illustrated by comparison with the literature data of five organisms related to strains tested in this study (Table 4). In case of *S. sanguis* ATCC 10556 / NCTC 7863 / HG 295 for example, the saliva-induced aggregation may have been dependent, apart from the incubation time applied, on the culture medium used. Besides the different experimental conditions and source of saliva, bacterial strain differences due to continuous subculturing (Gibbons & Van Houte 1980) may be responsible as well for dissimilar aggregation results.

In summary, optimal results with regard to the rate and titer of saliva-induced aggregation were obtained in this study when 200 μl of bacterial suspension, or 100 μl in case of interference by autoaggregation, after growth in GF-broth, was mixed with 100 μl of twofold serial dilutions of CHWS, and subsequently incubated in the presence of Ca-ions (1 mM) at 37° C up to 6 h.

In conclusion, most strains tested showed CHWS-induced aggregation, usually within 15 min to 3 h, dependent on the conditions mentioned. No obvious correlation, however, could be detected between any species and aggregation pattern, nor between the aggregation rate and final titer (Table 3, Figs. 1–3). Therefore it is likely that the various bacteria are aggregated in a strain-dependent way by nonidentical mechanisms, or that different amounts of either bacterial surface receptors or salivary agglutinins are involved in case of identical mechanisms. Future studies will focus on the aggregation activity of glandular salivas and on specific aggregation factors therein.

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