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Differentiation among Closely Related Organisms of the *Actinobacillus-Haemophilus-Pasteurella* Group by Means of Lysozyme and EDTA

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Bacteriolysis in Tris-maleate buffer (0.005 M, pH 7.2) supplemented with EDTA (0.01 M) and hen egg white lysozyme (HEWL, 1.0 μg/ml) was set up to assist differentiation between the taxonomically closely related *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. *A. actinomycetemcomitans* was more sensitive to lysis in this system than *H. aphrophilus*. The standard method for bacteriolysis separated the 10 tested strains of *A. actinomycetemcomitans* into two groups (I and II) based on their lysis patterns, whereas the 7 strains of *H. aphrophilus* examined were homogeneous. In group I of *A. actinomycetemcomitans*, EDTA displayed a considerable lytic effect, which was not increased by supplementation with HEWL. In group II, the lytic effect of EDTA was much less, but HEWL had a considerable supplementary lytic effect. When the turbidity of *A. actinomycetemcomitans* (ATCC 29522) or *H. aphrophilus* (ATCC 33389) suspended in Tris buffer was monitored at close pH intervals (0.2) from pH 5.2. to 9.2, maximal lysis of ATCC 29522 occurred with EDTA at pH 8.0 and with EDTA-HEWL at pH 7.6, while ATCC 33389 lysed with EDTA at pH 9.0 and with EDTA-HEWL at pH 9.2. When other members of the family *Pasteurellaceae* (*Haemophilus influenzae* type b, *Haemophilus paraphrophilus*, *Pasteurella multocida*, *Pasteurella haemolytica*, and *Pasteurella ureae*) were included for comparison, the group I strains of *A. actinomycetemcomitans* were the most rapidly lysed by EDTA. *H. paraphrophilus* was the least sensitive of the gram-negative strains tested, but not as resistant as *Micrococcus luteus* (control). *M. luteus* was the organism most sensitive to lysozyme, followed by *P. ureae* and the group II strains of *A. actinomycetemcomitans*, while the group I strains of *A. actinomycetemcomitans*, *H. paraphrophilus*, and *P. haemolytica* were the least sensitive organisms.

Organisms of the *Actinobacillus-Haemophilus-Pasteurella* group, which constitute the family *Pasteurellaceae*, have assumed increasing clinical importance in recent years (25). Unfortunately, distinction between these confusingly similar organisms may be hard to make from conventional biochemical tests, and there may be good reason to question the generic separation of this group. Some species within the different genera may also be hard to distinguish; among them are *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, and *Haemophilus paraphrophilus*. Additional criteria should therefore be sought to assist in the separation of organisms within the *Actinobacillus-Haemophilus-Pasteurella* group. One approach might be to investigate differences in the cell wall composition of these organisms. We have recently demonstrated that organisms within the *Actinobacillus-Haemophilus-Pasteurella* group, except *H. aphrophilus*, contain D-glycero-D-*manno*-heptose in whole cells (4) and lipopolysaccharide (LPS) (3). In *Salmonella* spp., so-called replicative-form LPS linked to 2-keto-3-deoxyoctulosonic acid-lipid A contains D-glycero-D-*manno*-heptose. Such mutants synthesize LPS with an incomplete core (14). Bacterial mutants with incomplete LPS are sensitive to a number of antibiotics usually restricted from their parent organisms (22). In *Escherichia coli* strains, reduced glucose residues in the polysaccharide portion of LPS are closely correlated with increased sensitivity of the cells to lysozyme (21).

The present study was carried out to determine whether the cell wall of *H. aphrophilus* is less permeable to bacteriolytic agents such as EDTA and lysozyme than are the cell walls of other organisms of the *Actinobacillus-Haemophilus-Pasteurella* group and whether bacteriolysis based on these agents can be used as an additional criterion for distinction among organisms of this vexed group.

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MATERIALS AND METHODS

Bacterial strains and growth. The strains of *Actinobacillus*, *Haemophilus*, *Pasteurella*, and *Micrococcus* (control) spp. used and their sources and sites of origin are given in Table 1. *Haemophilus influenzae* type b, strain B51, was obtained by T. Omland from H. C. Engbaek, Statens Seruminstitut, Copenhagen, Denmark). *Actinobacillus*, *Haemophilus*, and *Pasteurella* spp. were maintained anaerobically (80% N_2, 10% H_2, 10% CO_2) and *Micrococcus luteus* was maintained aerobically on blood or chocolate agar plates at 35 or 37°C, and these isolates were transferred weekly. Stock cultures were kept in liquid nitrogen after reconstitution from the lyophilized state. For assays of cell lysis, blood or chocolate agar plates inoculated with *Actinobacillus*, *Haemophilus*, or *Pasteurella* spp. were incubated anaerobically for 2 days at 35°C, and colonies were transferred on agar slants to preheated (37°C) brain heart infusion (Difco Laboratories, Detroit, Mich.) broth and cultured anaerobically at 35°C for 15.5 h. For *H. paraphrophilus* and *H. influenzae* type b, the broth was supplemented with filter-sterilized NAD (1 mg/ml) and hemin (5 mg/ml). *M. luteus* was cultured aerobically at 37°C for 3 days on blood agar plates and, thereafter, aerobically at 37°C for 6 h in brain heart infusion broth.

Lysozyme preparation. The preparation of lysozyme from fresh hen egg white (HEWL) has been described previously (11). The purified product, controlled by gel filtration and ultracentrifugation, had a molecular weight of 14,200 to

TABLE 1. List of bacteria investigated

Species and strain	Source	Site of origin
A. actinomycetemcomitans		
33384 (9710)[a]	ATCC[b] (NCTC)[c]	Lung abscess
29524	ATCC	Chest aspirate
29523	ATCC	Blood
29522	ATCC	Mandibular abscess
2112	FDC[d]	Periodontitis
2097	FDC	Periodontitis
2043	FDC	Periodontitis
511	FDC	Periodontitis
N27	FDC	Periodontosis
Y4	FDC	Periodontosis
H. aphrophilus		
33389 (5906)[a]	ATCC (NCTC)	Endocarditis
19415 (5886)	ATCC	Endocarditis
13252	ATCC	
655	FDC	Periodontitis
654	FDC	Periodontitis
626	FDC	Periodontitis
621	FDC	Periodontitis
H. paraphrophilus		
29241[a]	ATCC	Paronychia
29240	ATCC	Parietal abscess
H. influenzae type b		
33533	ATCC	Blood
31441	ATCC	Clinical isolate
B51	Omland (NDML)[e]	
P. multocida 10322[a]	NCTC	
P. ureae 10219[a]	NCTC	Ozena
P. haemolytica 9380[a]	NCTC	
M. luteus e4698 (2665)	ATCC (NCTC)	

[a] Type strain of species.
[b] American Type Culture Collection.
[c] National Collection of Type Cultures, London.
[d] Forsyth Dental Center, Boston, Mass.
[e] Norwegian Defense Microbial Laboratory, Oslo.

14,400. One unit produced a ΔA_{450} of 0.001/min at pH 6.24 at 25°C, with *Micrococcus lysodeikticus* (Sigma Chemical Co., St. Louis, Mo.) as the substrate in a 2.6-ml reaction mixture. The activity of the lysozyme preparation was approximately 43,000 U/mg of protein.

Turbidimetric assay of lysis. The susceptibility of the bacteria tested to EDTA and HEWL was analyzed turbidimetrically (9). Based on the standardized method, washed cells were suspended in 0.005 M Tris-maleate buffer (pH 7.2) (6), and the optical density (OD) was adjusted to approximately 1.0 at 540 nm, by using a Hitachi model 100-20 spectrophotometer (Hitachi, Ltd., Tokyo, Japan) before preincubation for 5 min in the buffer at 37°C in a Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). Thereafter, the OD was adjusted to 0.6 (approximately 9.1×10^8 cells per ml [1]), and 20 μl of buffer or lysozyme solution was added to the spectrophotometric cuvettes, each containing the bacteria suspended in 1,980 μl of 0.005 M Tris-maleate buffer supplemented with 0.01 M EDTA. The reduction in turbidity, referred to as cellular lysis, was recorded after 5, 10, 15, 20, 30, 40, and 50 min of incubation. In some experiments the assessment continued up to 3 h. All experiments were repeated from two- to sixfold with bacteria cultured at different time periods.

Transmission electron microscopic assessment of bacteriolysis. Selected bacterial strains were examined by transmission electron microscopy before and at the cessation of bacteriolysis. Lysis was stopped by adding a drop of 5 N hydrochloric acid to the bacterial suspension (10 ml), which was harvested immediately, washed, and fixed as described by Holt et al. (8). The pelleted cells were dehydrated in acetone and embedded in Vestopal W (Martin Jaeger Co., Geneva, Switzerland). Ultrathin sections, made on an ultrotome (LKB Instruments, Inc., Rockville, Md.) with glass knives, were stained with uranyl acetate and lead citrate and examined in a Philips EM400 electron microscope.

RESULTS

Lytic effect of the Tris-maleate buffer. The cellular lysis of *A. actinomycetemcomitans* (ATCC 29522) tended to be less in a 0.01 M than in a 0.005 M solution of Tris buffer supplemented with EDTA or EDTA-HEWL. With *H. aphrophilus* (ATCC 33389) there was no marked difference in the lytic effect of the supplemented buffers at either molarity. The 0.005 M buffer was used in further experiments. With no supplements in this buffer, there was a slight reduction in the turbidity of *A. actinomycetemcomitans* (ATCC 33384, ATCC 29522) and of *H. aphrophilus* (ATCC 33389) after incubation for 50 min. The reduction tended to be higher in *A. actinomycetemcomitans* than in *H. aphrophilus*.

Lytic effect of EDTA. By varying the concentration of EDTA through 0.001, 0.01, 0.05, and 0.1 M, we observed the highest lysis of *A. actinomycetemcomitans* (ATCC 33384, ATCC 29524, and ATCC 29522) in the Tris buffer supplemented with 0.01 M EDTA. This concentration of EDTA was used in further experiments. With *H. aphrophilus* (ATCC 33389) the difference in the lysis caused by the various EDTA concentrations was less marked. At most concentrations of EDTA tested, the lysis of *A. actinomycetemcomitans* ATCC 29524 and 29522 was higher with EDTA-HEWL than with EDTA. No such difference was observed with *A. actinomycetemcomitans* ATCC 33384.

Lytic effect of HEWL. By varying the HEWL concentration through 0.1, 1.0, 100.0, and 200.0 μg/ml, we found that *A. actinomycetemcomitans* (ATCC 29522) was considerably more sensitive to the enzyme than *H. aphrophilus* (ATCC 33389). HEWL at a concentration of 1.0 μg/ml yielded the highest lysis of *A. actinomycetemcomitans* and *H. aphrophilus* and was used in further experiments. With *A. actinomycetemcomitans*, lysozyme at 100.0 and 200.0 μg/ml caused a pronounced increase in the OD, possibly as a result of cellular aggregation. There was also an increase in the OD for *H. aphrophilus* at 200.0 μg/ml, but this increase was less and occurred later than in *A. actinomycetemcomitans*.

The addition of HEWL (1.0 μg/ml) to the Tris buffer (no EDTA present) did not cause higher lysis than did Tris alone, either in *A. actinomycetemcomitans* (ATCC 33384, ATCC 29522) or in *H. aphrophilus* (ATCC 33389). In *M. luteus* (ATCC e4698), however, a 50% reduction in the OD had occurred by 50 min after the addition of HEWL to the buffer.

Influence of substrate concentration on bacteriolysis. Bacteriolysis was also examined by varying the substrate

TABLE 2. Effect of EDTA (0.01 M) in Tris buffer (0.005 M)[a] on bacteriolysis

Bacterial strain[c]	Mean % of base-line OD[b] at incubation time (min)							
	0	5	10	15	20	30	40	50
A. actinomycetemcomitans								
Group I								
ATCC 33384	100	83.4	68.0	50.0	38.1	24.1	19.0	16.5
FDC 2112	100	79.4	56.3	40.6	33.1	29.2	25.6	23.1
FDC 511	100	88.4	72.4	55.0	44.5	31.7	25.0	20.7
FDC N27	100	96.7	93.0	89.0	81.5	69.0	58.0	45.7
Group II								
ATCC 29524	100	96.4	90.7	86.4	79.9	71.2	64.9	60.0
ATCC 29523	100	96.8	93.1	89.6	85.6	77.0	68.5	62.4
ATCC 29522	100	88.6	78.5	71.2	65.3	59.2	55.3	51.9
FDC 2097	100	93.2	86.2	80.2	74.8	68.0	62.0	58.0
FDC 2043	100	87.8	78.8	73.4	70.2	65.0	61.5	59.0
FDC Y4	100	95.2	85.7	76.6	69.7	59.6	54.3	51.5
H. aphrophilus								
ATCC 33389	100	98.1	95.2	93.4	91.7	86.9	83.3	78.0
ATCC 19415	100	94.3	92.3	88.0	85.0	79.7	73.8	67.7
ATCC 13252	100	96.7	94.2	92.6	90.6	86.8	82.5	79.8
FDC 655	100	96.6	94.8	92.8	92.0	87.2	82.0	78.1
FDC 654	100	96.5	93.2	90.7	88.0	79.8	74.3	70.2
FDC 626	100	97.0	94.2	92.1	88.8	83.4	77.8	73.8
FDC 621	100	95.7	93.0	90.1	87.0	81.0	74.5	70.0
H. paraphrophilus								
ATCC 29241	100	95.0	92.4	90.3	88.7	86.6	84.4	82.4
ATCC 29240	100	97.7	96.4	95.3	94.3	92.1	91.0	89.5
H. influenzae type b								
ATCC 33533	100	97.6	94.6	92.1	90.9	87.4	83.6	79.3
ATCC 31441	100	96.1	91.7	88.0	85.0	79.0	74.1	69.6
B51	100	92.7	84.5	78.6	73.8	65.4	60.3	57.8
P. multocida NCTC 10322	100	84.8	70.4	65.7	62.3	56.8	54.2	52.6
P. haemolytica NCTC 9380	100	88.0	79.6	73.5	69.7	64.5	61.0	58.8
P. ureae NCTC 10219	100	88.7	80.2	74.4	71.5	67.5	66.2	65.4
M. luteus ATCC e4698	100	98.2	97.3	96.1	95.4	94.5	94.0	93.1

[a] With unsupplemented buffer there was a slight reduction in turbidity after incubation of tested strains for 50 min (*A. actinomycetemcomitans* ATCC 33384 OD, 97.5%; ATCC 29522 OD, 96.2%; *H. aphrophilus* ATCC 33389 OD, 97.3%).

[b] Optical density at 540 nm.

[c] Source abbreviations are defined in the footnotes to Table 1.

concentration of *A. actinomycetemcomitans* (ATCC 29522) and *H. aphrophilus* (ATCC 33389) through initial ODs of 0.30, 0.45, 0.60, 0.75, and 1.00. In both *A. actinomycetemcomitans* and *H. aphrophilus*, the lowest OD after 50 min was recorded with EDTA and EDTA-HEWL when the lysis had been initiated at OD 0.60. Bacterial suspensions of OD 0.60 were used in the subsequent experiments.

Influence of temperature on bacteriolysis. When the incubation was performed at various temperatures (3, 13, 23, and 37°C), *A. actinomycetemcomitans* (ATCC 29522) was found to be considerably more sensitive to lysis than *H. aphrophilus* (ATCC 33389). In both species the highest lysis occurred at 37°C, which was used as the incubation temperature in further experiments. Irrespective of temperature, the lysis of *A. actinomycetemcomitans* (ATCC 29522) tended to be higher with EDTA-HEWL than with EDTA. *H. aphrophilus* (ATCC 33389) showed no marked difference in EDTA- and EDTA-HEWL-induced lysis at various temperatures.

Influence of pH on bacteriolysis. By varying the pH of the Tris buffer through pH 5.2, 7.2, and 8.4, we found that maximal lysis of *A. actinomycetemcomitans* (ATCC 33384, ATCC 29522) occurred at pH 7.2 and that of *H. aphrophilus* (ATCC 33389) occurred at pH 8.4. For a standard comparison of bacterial strains, Tris buffer at pH 7.2 was chosen. Since the lysis of *H. aphrophilus* seemed to increase with the pH of the buffer, the lysis of *H. aphrophilus* (ATCC 33389), as well as that of *A. actinomycetemcomitans* (ATCC 29522), was monitored at close pH intervals (0.2) within the range 5.2 to 9.2. With *A. actinomycetemcomitans* the greatest lysis after 50 min had occurred at pH 7.6 in the presence of EDTA-HEWL. For comparison, the highest lysis of *H. aphrophilus* with EDTA-HEWL was seen after 50 min at pH 9.2. With only EDTA as a supplement, maximal lysis of *A. actinomycetemcomitans* was recorded after 50 min at pH 8.0, while maximal disintegration of *H. aphrophilus* in the presence of EDTA occurred at pH 9.0.

Standardized lysis of *A. actinomycetemcomitans* and *H. aphrophilus*. The standardized test for bacteriolysis (see above) allowed the series of *A. actinomycetemcomitans* strains tested to be arranged in two groups based on their

TABLE 3. Effect of EDTA (0.01 M) and HEWL (1μg/ml) in Tris buffer (0.005 M)[a] on bacteriolysis

Bacterial strain[c]	Mean % of base-line OD[b] at incubation time (min)							
	0	5	10	15	20	30	40	50
A. actinomycetemcomitans								
Group I								
ATCC 33384	100	83.4	67.2	50.3	38.1	24.6	21.3	17.1
FDC 2112	100	85.8	53.3	39.2	31.7	26.8	23.6	21.2
FDC 511	100	86.6	69.2	53.0	43.7	31.8	24.6	20.2
FDC N27	100	96.7	92.6	87.2	80.5	68.0	60.0	48.2
Group II								
ATCC 29524	100	91.1	82.7	72.3	60.4	39.6	29.8	25.1
ATCC 29523	100	95.4	89.0	82.0	74.0	56.6	41.5	29.6
ATCC 29522	100	64.4	40.9	26.7	19.4	16.7	16.6	16.3
FDC 2097	100	81.4	65.6	53.0	44.0	34.7	31.3	30.3
FDC 2043	100	55.1	35.0	27.4	24.7	23.1	22.4	22.4
FDC Y4	100	91.3	73.8	58.9	46.6	31.2	24.6	20.4
H. aphrophilus								
ATCC 33389	100	98.1	95.5	92.9	91.4	86.3	81.0	73.4
ATCC 19415	100	94.7	91.2	86.0	81.2	75.0	68.2	61.0
ATCC 13252	100	95.9	94.7	90.9	88.6	79.8	74.4	69.3
FDC 655	100	96.7	94.6	92.0	89.0	83.0	78.3	74.3
FDC 654	100	96.8	94.0	91.4	88.0	78.0	72.3	68.1
FDC 626	100	97.0	94.0	90.8	88.0	79.0	71.2	64.0
FDC 621	100	95.6	92.2	88.6	83.8	74.2	65.9	61.2
H. paraphrophilus								
ATCC 29241	100	94.0	91.4	89.2	87.7	85.0	82.8	80.3
ATCC 29240	100	97.3	95.6	94.1	92.1	89.2	87.0	85.0
H. influenzae type b								
ATCC 33533	100	96.1	93.3	90.5	88.4	83.0	76.2	68.4
ATCC 31441	100	94.3	88.2	83.2	79.8	72.3	65.5	59.5
B51	100	85.2	76.1	68.0	65.5	57.8	54.3	53.0
P. multocida NCTC 10322	100	68.6	57.2	52.0	48.1	43.1	40.7	39.2
P. haemolytica NCTC 9380	100	87.7	78.8	73.0	68.6	62.8	58.7	56.2
P. ureae NCTC 10219	100	77.0	61.2	49.8	42.1	31.3	28.2	27.2
M. luteus ATCC e4698	100	54.0	27.4	11.1	6.5	1.5	0.8	0.2

[a] With unsupplemented buffer there was a slight reduction in turbidity after incubation of tested strains for 50 min (*A. actinomycetemcomitans* ATCC 33384 OD, 97.5%; ATCC 29522 OD, 96.2%; *H. aphrophilus* ATCC 33389 OD, 97.3%).
[b] Optical density at 540 nm.
[c] Source abbreviations are defined in the footnotes to Table 1.

lysis patterns. The strains of group I (ATCC 33384, Forsyth Dental Center [FDC] 2112, FDC 511, FDC N27) were highly to moderately sensitive to EDTA (Table 2). Lysozyme had no additional lytic effect on these organisms (Table 3). In group II (ATCC 29524, ATCC 29523, ATCC 29522, FDC 2097, FDC 2043, and FDC Y4), the cell-lysing effect of EDTA was lower than in group I (Table 2). On the other hand, lysozyme had a considerable supplementary effect on the cell lysis of the group II strains (Table 3). Contrary to the strains of *A. actinomycetemcomitans* tested, the strains of *H. aphrophilus* could not be arranged in distinct groups, even if there was a tendency towards higher cellular lysis with time in the *H. aphrophilus* strains when exposed to EDTA-HEWL (Table 3) compared with exposure to EDTA alone (Table 2). After EDTA-HEWL exposure for 3 h, the OD of *H. aphrophilus* (ATCC 33389) had dropped to 53.8% of the base line, whereas the OD of *A. actinomycetemcomitans* (ATCC 29522) was at 19.0%, i.e., approximately the level reached after 50 min.

Standardized lysis of other organisms within the family *Pasteurellaceae*. When the EDTA- and EDTA-HEWL-inducedlysis of other species within the family *Pasteurellaceae* was compared with that of *A. actinomycetemcomitans* and *H. aphrophilus* under standardized conditions, graded patterns of variation in susceptibility to lysis appeared. The group I strains of *A. actinomycetemcomitans* were the organisms which were most rapidly lysed by EDTA (Table 2). Also, the group II strains of *A. actinomycetemcomitans* and *Pasteurella* spp. tended to be more sensitive to EDTA than were strains of *H. aphrophilus*. *H. influenzae* type b and *H. aphrophilus* contained strains with overlapping EDTA sensitivity. *H. paraphrophilus* was the least sensitive to EDTA of the gram-negative organisms tested. *M. luteus* was even less affected by EDTA than *H. paraphrophilus*.

The combined lytic effect of EDTA and HEWL is shown in Table 3. Lysis of *M. luteus* occurred rapidly and was higher than that of the most sensitive gram-negative bacteria tested, which were *A. actinomycetemcomitans*, *P. ureae*, and *P. multocida*. *P. haemolytica-H. influenzae* type b and

TABLE 4. HEWL-induced bacteriolysis

Bacterial strain	% OD[a] in Tris buffer (0.005 M) supplemented with:				$\bar{x}_3$ ($\bar{x}_1 - \bar{x}_2$)[b]
	EDTA (0.01 M)		EDTA-HEWL (1μg/ml)		
	$\bar{x}_1$	Range	$\bar{x}_2$	Range	
A. actinomycetemcomitans					
Group I					
ATCC 33384	16.5	0.9	17.1	3.2	−0.6
FDC 2112	23.1	2.1	21.2	4.3	1.9
FDC 511	20.7	2.1	20.2	1.6	0.5
FDC N27	45.7	5.8	48.2	10.1	−2.5
Group II					
ATCC 29524	60.0	1.9	25.1	8.7	34.9
ATCC 29523	62.4	7.6	29.6	19.3	32.8
ATCC 29522	51.9	9.7	16.3	1.4	35.6
FDC 2097	58.0	6.0	30.3	2.3	27.7
FDC 2043	59.0	2.0	22.4	1.4	36.6
FDC Y4	51.5	1.4	20.4	5.2	31.1
H. aphrophilus					
ATCC 33389	78.0	9.5	73.4	12.8	4.6
ATCC 19415	67.7	5.8	61.0	7.1	6.7
ATCC 13252	79.8	5.0	69.3	6.5	10.5
FDC 655	78.1	1.8	74.3	0.3	3.8
FDC 654	70.2	4.0	68.1	1.8	2.1
FDC 626	73.8	11.7	64.0	18.8	9.8
FDC 621	70.0	9.3	61.2	12.8	8.8
H. paraphrophilus					
ATCC 29241	82.4	8.0	80.3	9.7	2.1
ATCC 29240	89.5	4.5	85.0	1.7	4.5
H. influenzae type b					
ATCC 33533	79.3	5.7	68.4	5.8	10.9
ATCC 31441	69.6	3.1	59.5	10.6	10.1
B51	57.8	2.4	53.0	3.7	4.8
P. multocida NCTC 10322	52.6	14.0	39.2	11.8	13.4
P. haemolytica NCTC 9380	58.8	15.8	56.2	16.0	2.6
P. ureae NCTC 10219	65.4	7.3	27.2	7.9	38.2
M. luteus ATCC e4698	93.1	7.9	0.2	0.3	92.9

[a] Optical density at 540 nm; organisms were incubated for 50 min.
[b] Actual HEWL-induced bacteriolysis.

H. influenzae type b-*H. aphrophilus* contained strains of overlapping sensitivity. *H. paraphrophilus* was the most resistant.

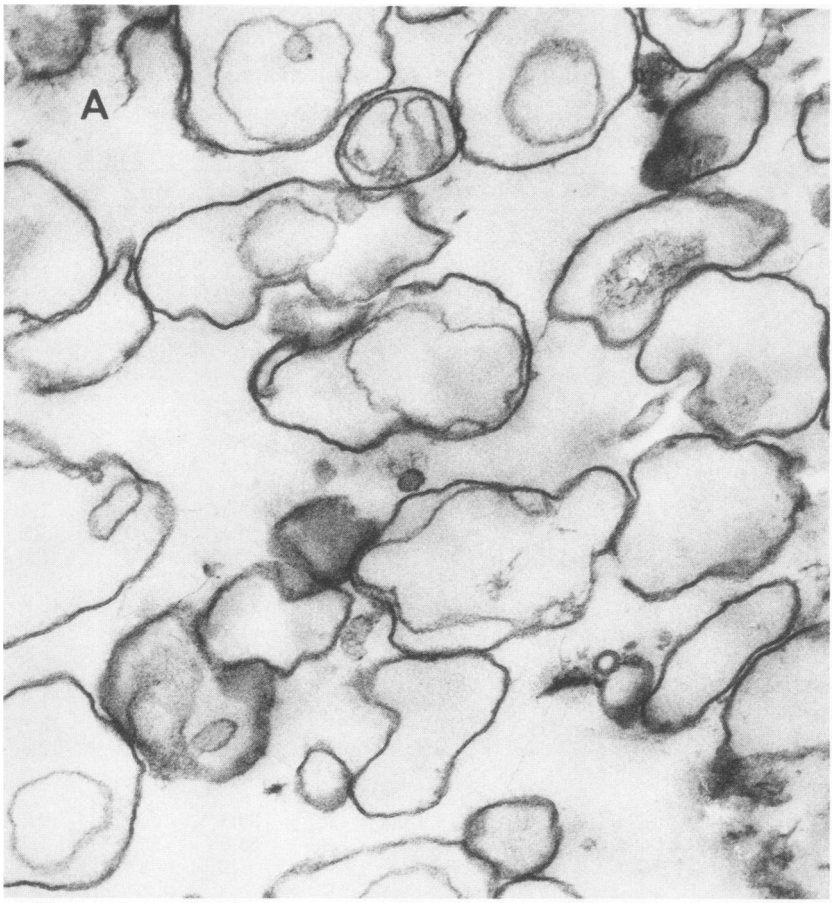
The lysozyme-induced lysis, measured as the difference in OD percentages after incubation of organisms for 50 min in buffer supplemented with EDTA and EDTA-HEWL, is shown in Table 4. *M. luteus* was more sensitive to lysozyme than any gram-negative organism examined. Among the latter, *P. ureae* and the group II strains of *A. actinomycetemcomitans* showed the highest lysozyme-induced lysis. The group I strains of *A. actinomycetemcomitans*, *H. paraphrophilus*, and *P. haemolytica* were least sensitive. *H. influenzae* type b and *H. aphrophilus* had strains with overlapping lysozyme sensitivity.

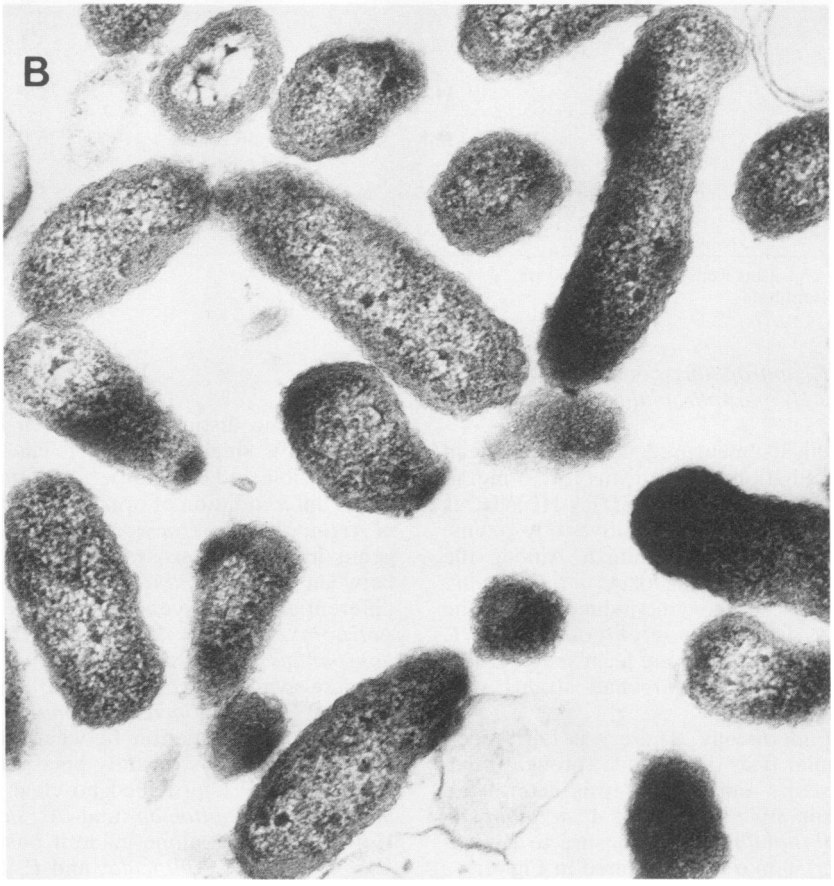
Transmission electron microscopy. There was fairly good agreement between cellular lysis determined through transmission electron microscopy and cellular lysis determined by turbidimetry. Representative pictures of *A. actinomycetemcomitans* and *H. aphrophilus* after exposure to EDTA-HEWL in Tris buffer for 50 min are presented in Fig. 1.

DISCUSSION

Taxonomic distinctions between bacteria should not be based on a single character. The lysis test, which was reproducible and easy to perform, may therefore only assist in the differentiation of organisms within the vexed grouping of *Actinobacillus*, *Haemophilus* and *Pasteurella* spp. On the genus level, the lysis test made it possible to differentiate between *Actinobacillus* and *Haemophilus*. It also allowed differentiation between closely related species such as *A. actinomycetemcomitans* and *H. aphrophilus* or *H. aphrophilus* and *H. paraphrophilus*. Relatively few characters are available in the routine laboratory for distinction between *A. actinomycetemcomitans* and *H. aphrophilus* (18), and differentiation between *H. aphrophilus* and *H. paraphrophilus* has recently been questioned (20, 23).

EDTA-HEWL provided no clear separation between *P. ureae* or *P. multocida* and *A. actinomycetemcomitans*. However, EDTA alone made it possible to differentiate *P. haemolytica*, *P. multocida*, and *P. ureae* from the group I


A


B

strains of *A. actinomycetemcomitans*. It has been suggested that *P. haemolytica* and *P. ureae* should be included in the genus *Actinobacillus* (5). This suggestion requires further consideration.

The present lysis system also made it possible to arrange strains of *A. actinomycetemcomitans* into two groups. The group I strains were strongly to moderately sensitive to EDTA, while HEWL provided no additional lysis. In the group II strains, EDTA had only a slight effect, while lysozyme caused considerable lysis. These differences have hitherto been unconsidered phenotypic characteristics of *A. actinomycetemcomitans*. Current genetic classifications (e.g., reference 23) have shown that members of lysis groups I and II are interrelated at very high DNA homology levels that do not allow discrimination of subspecies. The observed patterns of susceptibility to lysozyme and EDTA may therefore reflect infrasubspecific differences in the cell wall architecture. The graded patterns of variation in susceptibility to lysis in *A. actinomycetemcomitans* coincided with a relatively low content of rhamnose and fucose in whole cells from the group I strains and a higher content of these monosaccharides in the group II strains (2). Most of the group II strains, i.e., ATCC 29524, ATCC 29522, FDC 2097, FDC 2043, and FDC Y4, have been placed in the same serogroup (15). It should also be mentioned that ATCC 29524, ATCC 29522, and FDC Y4 of group II have been arranged in the same biotype (19).

H. aphrophilus was more resistant to lysis than *A. actinomycetemcomitans* but not so resistant as *H. paraphrophilus*. Therefore, the presence of D-glycero-D-*manno*-heptose did not necessarily reflect a reduced barrier function in *H. paraphrophilus* cells. Whether D-glycero-D-*manno*-heptose reflects core-deficient LPS in *A. actinomycetemcomitans*, *H. influenzae* type b, and *Pasteurella*, where it also has been detected (4), will have to be elucidated. The possibility cannot be excluded that D-glycero-D-*manno*-heptose in *Pasteurellaceae* reflects an LPS biosynthesis different from that of *Salmonella* spp., rather than an LPS deficient in function.

Light (unpublished data) and transmission electron microscopy confirmed that the reduction in turbidity was related to cellular lysis. There was a slight reduction in the turbidity of bacterial cells in the presence of Tris buffer alone (pH 7.2). Tris increases the permeability of the outer membrane in gram-negative bacteria (10, 26).

Also EDTA reduced the turbidity of suspensions of the gram-negative cells. Being a chelating agent, EDTA probably bound to Mg^{2+} or Ca^{2+}, which are the predominant ions of the cell wall envelope (24). Tris, which in itself chelates metals, may have interacted with EDTA to potentiate the binding of divalent ions. With atomic absorption spectrometry we have measured a considerable release of Ca^{2+} and particularly of Mg^{2+} from *A. actinomycetemcomitans* (ATCC 29522) and *H. aphrophilus* (ATCC 33389) cells after exposure to EDTA and EDTA-HEWL. The release was highest from *A. actinomycetemcomitans* exposed to EDTA-HEWL. Preexperimentally, the cellular concentration of Ca^{2+} was considerably higher than that of Mg^{2+} in both organisms. It is likely that particularly Mg^{2+} holds LPS in place in *A. actinomycetemcomitans* and *H. aphrophilus* and that its removal dissociates part of the LPS molecule from the cell wall, thereby contributing to the reduced turbidity (13). The increase in permeability caused by EDTA is supposed to be rather general, permitting passive diffusion of a number of substances normally excluded from gram-negative cells (13). EDTA may also affect the cytoplasmic membrane or other envelope structures of the bacterial cells (9), and long exposure may result in RNA breakdown and cell death, possibly because EDTA chelates divalent ions necessary to maintain ribosomal integrity and enzyme function (12). It was apparent that EDTA not only increased cell permeability but also initiated lysis. EDTA also activates phospholipase A, which weakens hydrophobic interactions between phospholipids and LPS (7). The EDTA breakdown of bacterial cells is thought to be most efficient in Tris buffer at an alkaline pH (13). The fact that the lysis of *H. aphrophilus* (ATCC 33389) was negligible in the buffer alone at pH 9.0, when maximal EDTA-induced lysis occurred, and at pH 9.2 with maximal EDTA-HEWL-induced lysis indicated a high lytic effect of EDTA on this organism at alkaline pH values. With *A. actinomycetemcomitans* (ATCC 29522), maximal lysis occurred with EDTA at pH 8.0 and with EDTA-HEWL at pH 7.6. At these pH levels the lytic effect of the buffer on *A. actinomycetemcomitans* was slight to moderate. It was considerable, however, at pH 9.0, indicating that the buffer rather than EDTA caused lysis of *A. actinomycetemcomitans* at this pH.

At pH 7.2 lysozyme alone caused a marked lysis in the control organism *M. luteus*, whereas *A. actinomycetemcomitans* (ATCC 33384, ATCC 29522) and *H. aphrophilus* (ATCC 33389) were rather unaffected. This suggested that EDTA is no prerequisite for lysozyme action on gram-positive cells, but that it is a prerequisite in gram-negative cells. Nevertheless, EDTA also seemed to promote the entry of lysozyme in gram-positive cells. There was a wide pH range at which lysozyme increased cell lysis in *A. actinomycetemcomitans*. Lysozyme lysed coliform bacteria within the pH range 3.5 to 10.0 (16). Lysozyme is an *N*-acetylmuramide glucanhydrolase (EC 3.2.1.17). It acts on peptidoglycan, hydrolyzing a specific glycoside bond, 4-0-β-(*N*-acetylmuramosyl)-*N*-acetylglucosamine, in the bacterial cell wall (17). The present results with *A. actinomycetemcomitans* suggested differences in the amount or in the framework of the peptidoglycan present in the cell walls of the two groups of strains. The variation in peptidoglycan framework is presently, with a few exceptions, of taxonomic value only in gram-positive microorganisms.

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FIG. 1. Comparative lysis in (a) *A. actinomycetemcomitans* (ATCC 29522) and (b) *H. aphrophilus* (ATCC 33389) after exposure to EDTA (0.01 M)-HEWL (1.0 μg/ml) in Tris-maleate buffer (0.005 M, pH 7.2) for 50 min at 37°C. Transmission electron microscopy; ×51,000.

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