JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1993, p. 2244–2247
0095-1137/93/082244-04$02.00/0

Vol. 31, No. 8

Comparison of Extracellular Enzymes of *Fusobacterium necrophorum* subsp. *necrophorum* and *Fusobacterium necrophorum* subsp. *funduliforme*

KINGSLEY KWAKU AMOAKO, YOSHITAKA GOTO, AND TOSHIHARU SHINJO*

Department of Veterinary Microbiology, Faculty of Agriculture, Miyazaki University, Miyazaki 889-21, Japan

Received 19 April 1993/Accepted 14 May 1993

A total of 10 strains each of *Fusobacterium necrophorum* subsp. *necrophorum* and *Fusobacterium necrophorum* subsp. *funduliforme* were tested for the production of 13 extracellular enzymes. DNase, alkaline phosphatase, and lipase were predominantly associated with all the strains of *F. necrophorum* subsp. *necrophorum*, with DNase not detected in any of the strains of *F. necrophorum* subsp. *funduliforme*. In addition, the strains of *F. necrophorum* subsp. *necrophorum* were generally more hemolytic than those of *F. necrophorum* subsp. *funduliforme*. Lecithinase, beta-lactamase, elastase, hyaluronidase, chondroitin sulfatase, and coagulase were not detected in any of the strains. DNase may be used to differentiate between the two subspecies.

Fusobacterium necrophorum has recently been classified into two subspecies as *Fusobacterium necrophorum* subsp. *necrophorum* and *Fusobacterium necrophorum* subsp. *funduliforme* (13). The strains of the former subspecies, which produces both hemolysin and hemagglutinin, have been found to be more pathogenic than those of the latter, which produces only hemolysin (12, 14). Reports also indicate that strains of *F. necrophorum* subsp. *necrophorum* are isolated mainly from pathological lesions of animals (6), whereas those of *F. necrophorum* subsp. *funduliforme* are mainly associated with the intestinal flora (5). The colonial, morphological, and biochemical differences between the two subspecies have been previously reported (13, 14). Lipase production has also been detected in *F. necrophorum* subsp. *necrophorum* and used as a criterion for the classification of the two subspecies (13). However, the extracellular enzyme profile of these two subspecies has not yet been documented. Knowledge about the production of extracellular enzymes by the two subspecies, apart from presenting distinguishing features for their differentiation, would also provide a firm basis for studies on their virulence mechanisms. In this study, we examined the spectrum of extracellular enzymes produced by the two subspecies. A total of 10 strains each of *F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme* were tested for the production of 13 extracellular enzymes.

The list and sources of the strains are as shown in Table 1. The criteria for the classification of these strains have been previously reported (13, 14). Hemolysin production was tested by the plate method. The strains were inoculated onto GAM (Gifu Anaerobic Medium) agar (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 3% horse erythrocytes. The composition of GAM agar is as follows: peptone, 1%; soya-bean peptone, 0.3%; Proteose Peptone, 1%; digested blood powder, 1.35%; yeast extract, 0.5%; meat extract, 0.22%; liver extract powder, 0.12%; glucose, 0.3%; potassium dihydrogen phosphate, 0.25%; sodium chloride, 0.3%; soluble starch, 0.5%; cysteine monohydrochloride, 0.03%; sodium thioglycolate, 0.03%; and agar 1.5%; the pH was 7.2, and the agar was sterilized at 115°C for 15 min. The plates were incubated in an anaerobic jar with a 10% carbon dioxide atmosphere at 37°C for 72 h by the steel wool method. About 10.6 g of steel wool dipped and squeezed in 20 ml of copper sulfate solution was used for a jar containing six plates. Clearing of zones around colonies of growth indicated hemolysis.

The method of Cowan (1) was used for the production of alkaline phosphatase. Briefly, 1% phenolphthalein phosphate solution was added aseptically to GAM agar, mixed thoroughly, and poured into plates. The organisms were inoculated as streaks, and after incubation by the steel wool method as described above, 0.1 ml of ammonia solution (28%) was placed on the lid of the petri dish and the medium was inverted above it. A bright pink coloration of colonies observed within 10 s indicated phosphatase production. *Staphylococcus aureus* served as the positive control.

DNase production was tested by using DNase agar (Difco Laboratories, Detroit, Mich.) supplemented with 0.1% sodium thioglycolate–0.05% cysteine monohydrochloride–0.5% yeast extract–0.05% soluble starch–0.2% glucose–1% Proteose Peptone no. 3–0.5% beef extract–5% blood digest. This was adjusted to pH 7.2 and autoclaved at 115°C for 15 min. The test organisms were inoculated as streaks on the plates and incubated as described above. After incubation, the plates were flooded with 1 N hydrochloric acid, and a clear zone around the streaks indicated DNase production (9). *S. aureus* was used as the positive control.

Lipase and lecithinase production was tested by the egg yolk agar plate method (4). Briefly, egg yolk saline solution (1:1) was added to GAM agar at a concentration of 10% and the strains were inoculated as streaks and incubated as described above. The formation of an oily iridescent sheen over and immediately around growth indicated lipase production. *Clostridium sporogenes* was used as the positive control. The production of a cloudy opaque zone in medium around growth, however, indicated lecithinase production. *Clostridium perfringens* was used as the positive control in this case.

Beta-lactamase production was assayed by incorporating

* Corresponding author.

TABLE 1. Strains of *F. necrophorum* used in this study

Subspecies	Strain	Source
F. necrophorum subsp. *necrophorum*	VPI 2891	W. E. C. Moore[a]
	N 167	M. Kanoe[b]
	Fn 43	Bovine liver abscess
	Fn 47	Bovine rumen fluid
	NCTC 10576	National Collection of Type Cultures
	SPH 29	B. Aalbaek[c]
	Fn 138	Calf pneumonia
	Fn 152	Bovine septicemia
	Fn 161	Bovine lumbar vertebra abscess
	Fn 69	Bovine liver abscess
F. necrophorum subsp. *funduliforme*	VPI 6161	M. Kanoe
	Fn 45	Bovine liver abscess
	No. 606	N. A. Kuck[d]
	No. 1260	N. A. Kuck
	SPH 28	B. Aalbaek
	SPH 6	B. Aalbaek
	SPH 11	B. Aalbaek
	Fn 49	Bovine rumen fluid
	Fn 20	Bovine liver abscess
	Fn 52	Heifer mastitis

[a] Virginia Polytechnic Institute and State University, Blacksburg.
[b] Yamaguchi University, Yamaguchi, Japan.
[c] Royal Veterinary and Agricultural University, Copenhagen, Denmark.
[d] American Cyanamid Company, Rahway, N.J.

cephaloridine (no. C-3519; Sigma Chemical Co., St. Louis, Mo.), a beta-lactam antibiotic, into GAM agar medium. The medium was autoclaved at 115°C for 15 min and cooled to 50°C, and this cephalosporin drug was added aseptically to give a final concentration of 0.5 μg/ml (3). The strains were plated as streaks and incubated anaerobically at 37°C for 72 h by the steel wool method. Visible growth indicated beta-lactamase production. *Bacteroides fragilis* served as the positive control.

Skim milk (Difco Laboratories) was used as the substrate to detect the production of caseinase. GAM agar was prepared to a double strength and autoclaved at 115°C for 15 min. An equal volume of 50% skim milk solution was also prepared and sterilized at 105°C for 10 min. After both preparations were cooled to 50°C, the skim milk solution was added to the GAM agar to give a final concentration of 25%. The strains were then plated as streaks and incubated anaerobically as described above for at least 1 week. Clearing of the milk medium around streaks indicated production of caseinase. *C. sporogenes* served as the positive control.

Hyaluronidase and chondroitin sulfatase production was examined by the method of Smith and Willet (15). The basal medium was GAM broth to which 1% Noble agar (Difco Laboratories) was added. Aqueous solutions of 2 mg of hyaluronic acid per ml containing chondroitin sulfate (Sigma no. 1876) and 5% bovine albumin fraction V were filter sterilized. The substrate and the albumin solutions were added to give final concentrations of 400 μg/ml and 1%, respectively, with a pH of 7.2. The plates were inoculated by making single streaks of the test strains, and after being incubated anaerobically as described above for 7 days, they were flooded with 2 N acetic acid for 10 min. This precipitated the nondegradable substrate as a conjugate with the albumin, leaving a clear zone around those colonies which produced soluble enzymes that attacked the hyaluronate and chondroitin sulfate. *C. perfringens* was used as the positive indicator.

Elastase production was ascertained as described elsewhere (17). The test strains were inoculated as streaks on GAM agar supplemented with 0.3% elastin (Sigma no. E-1625) and incubated for 2 weeks as described above. The clearing of zones around growth indicated elastase production. *Pseudomonas aeruginosa* served as the positive control.

Collagenase activity was examined by the method described by Krepel et al. (7). Briefly, 5 mg of type I collagen (Sigma no. C-9879) from bovine Achilles tendon was added to 5 ml of GAM broth in tubes and autoclaved. The tubes were then inoculated with the test strains and incubated anaerobically by the steel wool method described above for 2 weeks. The tubes were examined visually for collagen digestion at the end of the incubation period. A negative control without inoculum was also prepared, and *C. perfringens* served as the positive control.

The slide glass method was used for the detection of coagulase production. A colony of each strain grown on GAM agar was picked with a wire loop, and with minimum spreading, the colony was emulsified in a drop of water on a microscope slide. The bacterial suspension formed was then stirred with a straight wire which had been previously dipped into rabbit plasma. The formation of macroscopic clumping within 5 s indicated a positive reaction. *S. aureus* served as the positive control.

The liquefaction of gelatin was tested as previously described (14) by inoculating strains into medium supplemented with 1.2% gelatin (Difco Laboratories). Tubes were incubated anaerobically as described above for 2 weeks and placed at 4°C for about 20 min, after which they were examined for medium liquefaction. *C. perfringens* served as the positive control.

The results of the enzyme spectrum examined are shown in Table 2. Enzymes most frequently detected were hemolysin, phosphatase, DNase, lipase, and gelatinase. None of the strains produced all of the 13 enzymes. In general, strains belonging to *F. necrophorum* subsp. *necrophorum* produced wider zones of hemolysis (4 to 6 mm) than those of *F. necrophorum* subsp. *funduliforme* (1 to 3 mm). The production of DNase was found to be subspecies dependent. All the strains of the former subspecies tested positive for this enzyme, with all those of the latter being negative.

We have shown that there is great variability among the strains of the two subspecies of *F. necrophorum* in regard to their ability to produce extracellular enzymes. In general, strains of *F. necrophorum* subsp. *necrophorum* were quite active enzymatically, producing more of the enzymes for which assays were performed. The strains of this subspecies have been found to be more pathogenic than those of *F. necrophorum* subsp. *funduliforme* (12, 14). Lipase production has been used as a criterion for the classification of the two subspecies of *F. necrophorum* (13), although this was not clearly indicated from the results of this study. Extracellular DNase production has been examined for *Fusobacterium* species (9). The results of the present study showed that the strains within the two subspecies were clearly distinguished with respect to the production of this enzyme, and the fact that only strains of *F. necrophorum* subsp. *necrophorum* produced DNase may suggest a differential criterion for *F. necrophorum*.

Alkaline phosphatase production has been observed for some anaerobic bacteria (10). In the present study, strains within the two subspecies of *F. necrophorum* were some-

TABLE 2. Extracellular enzymes of two subspecies of *F. necrophorum*

Strain	Enzyme activity[a]												
	Hem	Phos	DNase	Lip	Lec	β-Lac	Cas	Hya	Elas	Coll	Chon	Coag	Gel
F. necrophorum subsp. *necrophorum*													
VPI 2891	+	+	+	+	−	−	W	−	−	+	−	−	+
N 167	+	+	+	+	−	−	−	−	−	−	−	−	+
Fn 43	+	+	+	+	−	−	−	−	−	+	−	−	+
NCTC 10576	+	+	+	+	−	−	−	−	−	+	−	−	+
Fn 47	+	+	+	+	−	−	−	−	−	+	−	−	+
SPH 29	+	+	+	+	−	−	W	−	−	−	−	−	+
Fn 138	+	+	+	+	−	−	W	−	−	−	−	−	+
Fn 152	+	+	+	W	−	−	W	−	−	W	−	−	−
Fn 161	+	+	+	+	−	−	−	−	−	+	−	−	+
Fn 69	+	+	+	+	−	−	−	−	−	+	−	−	−
F. necrophorum subsp. *funduliforme*													
VPI 6161	+	−	−	−	−	−	−	−	−	W	−	−	+
No. 606	+	+	−	−	−	−	−	−	−	W	−	−	+
No. 1260	+	+	−	−	−	−	−	−	−	W	−	−	−
SPH 6	+	−	−	−	−	−	−	−	−	W	−	−	−
SPH 11	+	−	−	W	−	−	−	−	−	−	−	−	−
Fn 49	+	−	−	+	−	−	−	−	−	−	−	−	+
Fn 20	+	−	−	W	−	−	−	−	−	+	−	−	−
Fn 52	+	−	−	−	−	−	−	−	−	−	−	−	−
Fn 45	+	−	−	+	−	−	−	−	−	+	−	−	−
SPH 28	+	−	−	+	−	−	−	−	−	−	−	−	−

[a] Hem, hemolysin; Phos, phosphatase; Lip, lipase; Lec, lecithinase; β-Lac, β-lactamase; Cas, caseinase; Hya, hyaluronidase; Elas, elastase; Coll, collagenase; Chon, Chondroitin sulfatase; Coag, coagulase; Gel, gelatinase; +, positive reaction; −, negative reaction; W, weak reaction.

what distinguished with respect to the production of this enzyme. Proteases like collagenase and gelatinase have been shown to be present in anaerobic bacteria isolated from human clinical specimens (16). Gelatinase has been previously shown to be present in some strains of the two subspecies (14). Enzymes like hyaluronidase, lecithinase, chondroitin sulfatase, beta-lactamase, and elastase were not detected in any of the strains tested. These enzymes have, however, been reported for other anaerobic bacteria (11, 16, 17). The present study indicated that strains of *F. necrophorum* subsp. *necrophorum* are more hemolytic than those of *F. necrophorum* subsp. *funduliforme*, and this is consistent with a previous report (2).

The findings of this study show that the production of DNase, alkaline phosphatase, and lipase was predominantly associated with *F. necrophorum* subsp. *necrophorum*; in particular, the production of DNase, which represents the first report on the two subspecies, may add to the existing knowledge on the differential features of the two subspecies. Phosphatase and lipase have been found to be associated with the virulence of some anaerobic bacteria (8, 10) and thus may be potential virulence factors enhancing the pathogenicity of *F. necrophorum* subsp. *necrophorum*. Hence, this may be a first step towards the elucidation of the role of these enzymes as enzymatic virulence factors. Furthermore, this study consists of a collection of simple, effective, and less expensive methods that anaerobic bacteriologists who are practicing without the available commercial kits may find useful.

REFERENCES

1. **Cowan, S. T.** 1974. Cowan and Steel's manual for the identification of medical bacteria, 2nd ed., p. 178. Cambridge University Press, Cambridge.
2. **Fiévez, L.** 1963. Etude comparee des souches de sphaerophorus necrophorus isolees chez l'homme et chez l'animal. Presses Academiques Europeennes, Brussels.
3. **Hackman, A. S., and T. D. Wilkins.** 1975. Comparison of cefoxitin and cephalothin therapy of a mixed *Bacteroides fragilis* and *Fusobacterium necrophorum* infection in mice. Antimicrob. Agents Chemother. **8**:224–225.
4. **Holdeman, L. V., P. E. Cato, and W. E. C. Moore.** 1977. Anaerobe laboratory manual, 4th ed., p. 126. Virginia Polytechnic Institute and State University, Blacksburg.
5. **Kanoe, M., H. Imagawa, and M. Toda.** 1975. Distribution of *Fusobacterium necrophorum* in bovine alimentary tracts. Bull. Fac. Agric. Yamaguchi Univ. **26**:161–172.
6. **Kanoe, M., Y. Izuchi, M. Kemi, M. Toda, and Y. Hara.** 1979. Hepatic abscess in fattened dairy steers. Jpn. J. Vet. Sci. **41**:73–76.
7. **Krepel, J. C., M. C. Gohr, C. E. Edmiston, and G. S. Farmer.** 1991. Anaerobic pathogenesis: collagenase production by *Peptostreptococcus magnus* and its relationship to site of infection. J. Infect. Dis. **163**:1148–1150.
8. **Lee, W. L., A. R. Shalita, K. Suntharalingam, and S. M. Fikrig.** 1982. Neutrophil chemotaxis by *Propionibacterium acnes* lipase and its inhibition. Infect. Immun. **35**:71–75.
9. **Porschen, R. K., and S. Sonntag.** 1974. Extracellular DNase production by anaerobic bacteria. Appl. Microbiol. **27**:1031–1033.
10. **Porschen, R. K., and E. H. Spaulding.** 1974. Phosphatase activity of anaerobic organisms. Appl. Microbiol. **27**:744–747.
11. **Rowland, M. D., V. E. Del Bene, and J. W. Lewis.** 1987. Factors affecting antimicrobial susceptibility of *Fusobacterium* species. J. Clin. Microbiol. **25**:476–479.
12. **Shinjo, T.** 1983. *Fusobacterium necrophorum* isolated from a hepatic abscess and from mastitic udder secretions in a heifer. Ann. Microbiol. (Inst. Pasteur) **134B**:401–409.
13. **Shinjo, T., T. Fujisawa, and T. Mitsuoka.** 1991. Proposal of two subspecies of *Fusobacterium necrophorum* (Flügge) Moore and Holdeman: *Fusobacterium necrophorum* subsp. *necrophorum* subsp. nov., nom. rev. (ex Flügge 1886), and *Fusobacterium necrophorum* subsp. *funduliforme* subsp. nov., nom. rev. (ex

Hallé 1898). Int. J. Syst. Bacteriol. **41:**395–397.
14. **Shinjo, T., S. Miyazato, C. Kaneuchi, and T. Mitsuoka.** 1981. Physiological and biochemical characteristics of *Fusobacterium necrophorum* biovar A and B strains and their deoxyribonucleic acid homology. Jpn. J. Vet. Sci. **43:**233–241.
15. **Smith, R. F., and N. P. Willet.** 1968. Rapid plate method for screening hyaluronidase and chondroitin sulfatase-producing microorganisms. Appl. Microbiol. **16:**1434–1436.
16. **Steffen, E. K., and D. J. Hentges.** 1981. Hydrolytic enzymes of anaerobic bacteria isolated from human infections. J. Clin. Microbiol. **14:**153–156.
17. **Stewart, D. J.** 1979. The role of elastase in the differentiation of Bacteroides nodosus infections in sheep and cattle. Res. Vet. Sci. **27:**99–105.