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Chemical and biological characterization of phenol-water extracts from Yersinia enterocolitica

Steven J. Wells
The University of Montana

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CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF

PHENOL-WATER _EXTRACTS FROM YERSINIA ENTEROCOLITICA

By

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ABSTRACT

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Chemical and Biological Characterization of Phenol-Water Extracts *mg n*
from <u>Yersinia enterocolitica</u> (71 pp.)

Director: Dr. M. J. Nakamura

Aqueous-phenol extraction of whole cells of two strains of <u>Yersinia enterocolitica</u> was undertaken. These strains were chosen for their biochemical, serological, and epidemiological differences. One strain was a human spleen, serotype O:8, rhamnose nonfermenting isolate, while the other was a water, serotype O:17, rhamnose fermenting isolate. Cells for extraction were cultivated at incubation temperatures of 25 and 37 C for each strain.

Identification of a sedimentable, water extractable lipopolysaccharide (LPS) from each strain at both growth temperatures was accomplished. Assays for chemical content included determinations of percent nitrogen, phosphorous, protein, lipid, total carbohydrate, total hexose, hexosamine, and 2-keto-3-deoxyoctonate (KDO). Biological assays included determinations of toxicity for mice and 11 day old chick embryos, pyrogenicity in rabbits, mitogenicity for murine spleen cells, and tumor suppression activity. The latter two assays were studied in several mouse strains. The chemical composition and biological activity of the LPS from the <u>Y. enterocolitica</u> strains showed that they resembled a similarly extracted LPS from <u>Escherichia coli</u>.

The data collected from these studies indicated two things. First, a taxonomical relationship of these strains to one another, to the Enterobacteriaceae, and to the genus <u>Yersinia</u> was established. This was displayed by the correspondence in extractability pattern, chemical composition, and biological activity of LPS from these strains to one another and to the LPS from <u>E. coli</u>. Secondly, although no strain dependent variation in LPS potency was observed, a temperature-dependent variation in LPS production was. More LPS was extracted from 25 C cultures of both strains than from their respective 37 C cultures. Since increased experimental pathogenic activity was reported in the literature for <u>Y. enterocolitica</u> strains grown at 25 C, the data from this study suggested that LPS, or endotoxin, may contribute to pathogenicity of <u>Y. enterocolitica</u> in clinical infection.

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ABBREVIATIONS

BHI	brain heart infusion
C	degrees centigrade
$CELD_{50}$	50% lethal dose in chick embryos
Con A	concanavalin A
DDH	dideoxyhexose
DNA	deoxyribonucleic acid
FAA	fatty acid amide
FAE	fatty acid ester
FI_{40}	dose producing a fever index of 40 centimeters squared
g	grams
ip	intraperitoneal
iv	intravenous
KDO	2-keto-3-deoxyoctonate
LD_{50}	50% lethal dose
LPS	lipopolysaccharide
M	molar
mg	milligrams
ml	milliliter
MLD_{50}	50% lethal dose in white mice
mM	millimolar
N	normal
nm	nanometers

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p	probability
PBBS	phosphate-buffered balanced salts
RNA	ribonucleic acid
rpm	revolutions per minute
sc	subcutaneous
ug	micrograms

CHAPTER I

INTRODUCTION

Yersinia enterocolitica is an ubiquitous, gram-negative, fermen-
tative coccobacillus which has evoked recent interest as a human
pathogen. Various strains were shown to be invasive and/or toxigenic
under varying conditions of cultivation of the organism and method
of testing for pathogenicity (7,26,63,76,98). However, Y. enteroco-
litica's mechanism of pathogenicity remains undetermined. This study
was designed to assess the possible contribution of endotoxin produc-
tion by Y. enterocolitica strains, selected for certain characteris-
tics and cultivated under various conditions, to pathogenicity.

The first isolation of Y. enterocolitica was described in 1939
(20). The organism, which resembled Yersinia pseudotuberculosis
biochemically and pathogenetically, was called Bacterium enterocoli-
ticum (16,20). Ten years later, the first isolation of the organism
in Europe was reported (19,22). Another fifteen years transpired
before it was associated with human disease (16). Prior to this,
some investigators studied strains of the bacterium morphologically,
biochemically, and serologically, determined that it was related to
Y. pseudotuberculosis and designated it Pasteurella X (16,20,22).
It also was called P. pseudotuberculosis type b and Germe X (16,22,
68,73). In contrast to Y. pseudotuberculosis, Y. enterocolitica
fermented cellobiose, sucrose, and sorbose, and decarboxylated orni-

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thine, but generally failed to ferment salicin or hydrolyze esculin (16,20,73). Yersinia pestis was readily distinguishable from both of these species on the basis of epidemiology, pathogenicity, serology, and biochemistry (20). In 1964, it was proposed that a new genus, Yersinia, be established and that the above three species be placed in it (20,22). The genus was placed in the family Enterobacteriaceae (22,31,73). The addition of two new species has been proposed, Yersinia philomiragia (19) and Yersinia ruckeri (37), but not yet accepted. Very little is known about either of these organisms.

Y. enterocolitica was organized into five biotypes by Nilehn (73) based upon biochemical characterisitics. Group 5 contained all those strains which did not fit in one of the other four biotypes. In the following year a similar classification system, substituting lecithinase activity for esculin and salicin in Nilehn's scheme, was devised (16,20). Both investigations demonstrated that many of the biochemical reactions were subject to temperature-dependent expression, a useful phenomenon in the differentiation of this species from other Yersinia and Enterobacteriaceae (20,73,90). Motility at 25 C but not at 37 C, presumably due to loss of flagella at the higher temperature (72), was particularly helpful in distinguishing this bacterium from others (20,31,46). Antigenically, Y. enterocolitica was separated into 34 serotypes based upon O somatic antigens and 19 serotypes based upon flagellar H antigens (73). Because of a close association of certain H and O antigens, only the latter usually have been reported in the literature (16,73). Phagetyping of European strains yielded 10 phage types, which were useful in distinguishing

between European and Canadian sources of serotype O:3 strains (93).
All isolates in the U. S. A. have been nontypable by bacteriophage
thus far (16,20,31). Unfortunately, there appears to be little cor-
relation between the biotyping schemes and serotyping, phagetyping,
strain virulence, or epidemiological repository.

Recently, however, the taxonomy of Y. enterocolitica has been
questioned on several levels. First, there was some controversy on
the inclusion of the genus Yersinia in the family Enterobacteriaceae.
This was a two pronged problem in that Y. enterocolitica apparently
demonstrated a stronger relationship to the Enterobacteriaceae than
did Y. pseudotuberculosis or Y. pestis. Investigations that were
pertinent to this dilemna included DNA hybridization, antigenic cross-
reactivity, and lipopolysaccharide (LPS) composition and structure
studies.

DNA hybridization studies conducted by Brenner et al. (19) demon-
strated a high degree of relatedness between each of the Yersinia
species and a distant, but acceptable, relationship to the Entero-
bacteriaceae. In contrast, Moore and Brubaker (71) found a strong
degree of relatedness of DNA from Y. enterocolitica, but not Y. pseudo-
tuberculosis or Y. pestis, with DNA from the Enterobacteriaceae.
They also showed that a weak DNA relationship between Y. enterocoli-
tica and the other two Yersinia species existed.

In immunological cross-reactivity studies on Yersinia the situa-
tion was not made any clearer. Some Y. enterocolitica strains were
reported to be antigenically related to Proteus morganii (101), Vibrio
cholerae (12,85), and Kauffmann-White group N Salmonella serotypes

(30). These studies did not test for such relationships with Y.
pseudotuberculosis or Y. pestis. Maeland and Digranes (64) found
that Y. enterocolitica possessed the common enterobacterial antigen,
a marker characteristic of the Enterobacteriaceae. They also found
one Y. pseudotuberculosis strain positive for this antigen. Barber
and Eylan (10,11) showed that whole cell antigen preparations from
Y. enterocolitica cross-reacted with Y. pestis, Salmonella, and Shi-
gella antisera. Also, similarly prepared Y. pestis antigens strongly
cross-reacted with Y. enterocolitica antisera, but only weakly cross-
reacted with Salmonella and Shigella antisera. Alonso et al. (6)
corroborated the antigenic relationship between Y. enterocolitica
and Y. pestis by demonstrating a marked resistance to infection by
Y. pestis in mice previously challenged with a large dose of an a-
virulent strain of Y. enterocolitica. In contrast, one strain of
Y. enterocolitica, M.Y. 79 serotype O:9, displayed a strong cross-
reactivity to Brucella species (32,48,67,85). This cross-reactivity
was ultimately shown to reside in the LPS (50,51,66). Subsequently,
the M.Y. 79 strain was shown to be unique among Y. enterocolitica
strains, since in 14 other strains, including several O:9s, none
possessed cross-reacting antigens to Brucella abortus (51).

Analyses of composition and structure of the LPS from Yersinia
species provided information relevant to the taxonomical relationship
of this genus to the Enterobacteriaceae. Kanamori (55) isolated LPS
extracts from two Y. enterocolitica strains by the aqueous-phenol
and trichloroacetic acid extraction methods whose biological acti-
vities and chemical compositions were similar to an identically ex-

tracted LPS from Escherichia coli. Acker (1) and Acker and Warten-
berg (2) demonstrated, in electron micrographs, that LPS obtained in
the aqueous phase in phenol-water extraction of Y. enterocolitica was
structurally similar to LPS from E. coli and Salmonella typhimurium.
In contrast, phenol-water extraction of the M.Y. 79 strain resulted
in recovery of LPS in the phenol phase (32,48,67). This LPS was simi-
lar in extractability pattern, chemical composition, and biological
activity to identically extracted LPS from B. abortus (49,50,66).
However, further investigations revealed a water extractable LPS from
the M.Y. 79 strain whose chemical and biological characteristics were
comparable to the phenol extracted LPS (49,52,66).

More detailed chemical analyses of the LPS from the different
Yersinia were accomplished. Hartley et al. (42) showed that LPS
from Y. pestis contained the same sugars generally found in the "core"
region of LPS from the Enterobacteriaceae. The lipid A from this or-
ganism contained virtually only D-3-hydroxytetradecanoic acid (42).
This fatty acid was common in lipid A of the Enterobacteriaceae (4,21).
Analyses of the sugar composition of LPS from Y. pseudotuberculosis
showed a slightly more complex content than was true of Y. pestis
(84,95). Among the sugars were unusual dideoxyhexoses (DDHs), which
were encountered only rarely in the LPS from other bacteria (84,95).
Lipid A from Y. pseudotuberculosis contained small, but significant,
quantities of dodecanoic and hexadecanoic acids in addition to D-3-
hydroxytetradecanoate (21,59). This also reflected a more complex
composition than was found in lipid A of Y. pestis. The chemical
composition of LPS from Y. enterocolitica, with the exception of the

M.Y. 79 strain, was similar to that from Y. pseudotuberculosis and the Enterobacteriaceae (13,55,103). Although no DDHs were recovered in the LPS from Y. enterocolitica, unique deoxyhexoses, such as 6-deoxy-L-altrose, were found (36,103). A variety of fatty acids in the lipid A of Y. enterocolitica were found. Chiefly recovered was D-3-hydroxytetradecanoate, with lesser amounts of dodecanoate, tetradecanoate, and hexadecanoate also detected (21,66). Finally, the LPS from each of the Yersinia species contained 2-keto-3-deoxyoctonate (KDO), a characteristic sugar in LPS from the Enterobacteriaceae (13,42,55,66,84).

The sum of the data from the DNA hybridization, antigenic relationship, and LPS composition studies suggests that Y. enterocolitica may be more closely related to the Enterobacteriaceae than are Y. pseudotuberculosis or Y. pestis. In addition, the relationship of these species to one another requires further clarification.

The second topic of taxonomic controversy concerned the subspeciation of Y. enterocolitica and the Y. enterocolitica-like organisms. Atypical rhamnose fermenting, citrate-positive strains and sucrose nonfermenting strains of Y. enterocolitica were described in several studies (17,28,40,43,88,94). Many of these organisms were nontypable by serology or by bacteriophage, but were serotypes O:16 or O:17 when they were typable (16,29,86). Chester et al. (29) demonstrated the immunological uniqueness of rhamnose-positive strains. These strains cross-reacted with one another but not with rhamnose-negative strains. Thus, it was suggested that a separate species, Yersinia rhamnophilica, containing these rhamnose fermenting strains

be formed (16,29). Brenner et al. (19) found at least three DNA-relatedness groups of Y. enterocolitica, each of which also could be biochemically defined. These groups corresponded to the typical Y. enterocolitica strains, indole-positive and indole-negative; rhamnose-positive and raffinose- and melibiose-negative strains; and rhamnose-, raffinose-, and melibiose-positive strains. They further postulated that a fourth DNA-relatedness group consisting of sucrose-negative strains may exist. On the basis of this evidence, two new species of rhamnose-positive organisms were proposed, Yersinia intermedium and Yersinia frederikseni (19,68). Acceptance of any of this additional speciation remains to be resolved.

Y. enterocolitica was shown to be capable of growth on a wide variety of substrates and over a fairly broad temperature range (28, 62,73). The organism was not fastidious since growth was possible in a minimal salt buffer with a single carbon source (62,87). Growth at 4, 25, and 37 C in pure culture was observed, but with long generation times (16,73). Since it was relatively slow growing, and because it did not compete well with other gram-negative, enteric bacteria in a mixed-flora environment, cold enrichment in various milieu was suggested for isolation of this organism from clinical and other sources (35,46,62,88). At present, little is known about the resistance of this bacterium to heat, cold, dessication, and NaCl (62).

Isolation of Y. enterocolitica from a wide variety of sources has been accomplished with increasing frequency. It was recovered from humans (9,39,74,107), other mammals, especially swine (93,108), cattle (108), rats and other rodents (56,57,96,108), and pets, such

as dogs and cats (96,104), birds (94), fish (57,62), amphibians (17),
and invertebrates (17,77). In foods, it was isolated from beef (41,
53), lamb (40,41), chicken (96), milk and dairy products (15,86,88),
fruit (62), and water (43,47,57,61,87). Transmission of the bacterium
to humans by water (61) and chocolate milk (15) was reported. Although
transmission of Y. enterocolitica to humans by meats was suspected,
no instance of it was observed (46,62,68). So, the natural reservoir
for this bacterium remains a mystery. Possibly, multiple reservoirs
exist. Indeed, a relationship between certain sources and specific
serotypes was suggested (56,93,104). Such information may be useful
in determining the route of transmission of the organism to man.

The increased isolation of Y. enterocolitica, clinically and en-
vironmentally, heightened awareness of the public health significance
of this organism. Toivanen et al. (92) questioned whether this reflects
a real increase in the incidence of disease due to Y. enterocolitica,
or whether it was the direct consequence of improved laboratory tech-
niques and diagnostic acumen in its recovery. Regardless, the inci-
dence increased worldwide (9,16,73,94,106). However, recognition of
specific serotypes was restricted geographically. In Europe and Asia,
the serotypes usually found in humans were 0:3 and 0:9 (9,74,91,106,107).
There was a greater diversity of human serotypes isolated in the U. S. A.,
although serotype 0:8 was found most frequently (31,39,46,58,94). In
Canada, the most common human serotypes were 0:3, 0:5,27, and 0:6,30,
the Canadian 0:3 being a different phage type than the European 0:3
(23,94). Other serotypes also were recovered from humans, including
atypical, rhamnose-positive Y. enterocolitica and Y. enterocolitica-

like organisms (16,46,68).

Studies recognized three major forms of clinical infection due to $Y.$ enterocolitica. First, it was regarded as a cause of mild to severe gastroenteritis, usually culminating in spontaneous recovery with no post-infectious sequelae (9,15,16,74,107). However, this position may require revaluation. Zen-Yoji and Maruyama (106) reported finding no carriers of $Y.$ enterocolitica in examination of more than 35,000 stools from healthy individuals. In contrast, Szita and Svidro (91) demonstrated that approximately 25% of all persons positive for $Y.$ enterocolitica were symptomless excreters. Since the recovery rate of $Y.$ enterocolitica in fecal specimens from individuals with enteric complaints was reported to be only about 1-2% (62,68), recovery of it in cases of gastroenteritis may not be significant. Second, $Y.$ enterocolitica was found in cases of mesenteric lymphadenitis and terminal ileitis, the symptoms sometimes mimicking acute appendicitis (3,18,39, 73,74). The pathology in such cases was virtually indistinguishable, grossly and histologically, from that produced by $Y.$ pseudotuberculosis (3,18,74). Also, mesenteric lymphadenitis produced experimentally in laboratory animals by $Y.$ enterocolitica was similar, in appearance and character, to that produced by $Y.$ pseudotuberculosis in animals and by $Y.$ enterocolitica in humans (25,99). Third, this bacterium was isolated in cases of septicemia or other severe conditions (16,58, 90). These cases, which were periodically fatal, generally occurred in the young, old, or immunocompromised (16,39,58,90). However, cases in otherwise healthy individuals also were reported (39,90). More recently, an increase in the frequency of post-yersionitic erythema

nodosum or polyarthritis, especially in females, was reported (16,74,90).

Initially, experimental models to study pathogenicity in labora-tory animals met with little success. Mollaret and Guillon (70) tested 106 strains of Y. enterocolitica and reported that they were unable to produce mortality in several animal species using a variety of routes of inoculation. Later, however, it was shown that a human serotype 0:8, the WA strain, was highly pathogenic for several mouse strains by oral, aerogenic, and parenteral routes of inoculation (24,25,26,27). Some strains also were pathogenic for athymic (5,7), axenic (14,25), irradiated (105), and cyclophosamide treated (7) mice by oral, aero-genic, intraperitoneal (ip), or intravenous (iv) routes of inoculation. In each of these studies, lethality, recorded as the 50% lethal dose (LD_{50}), was the usual measurement of pathogenicity. Mixed success was reported in production of experimental pathogenicity in uncompro-mised strains of mice. Carter and Collins (26) demonstrated a lower order of susceptibility to infection with Y. enterocolitica in either C57Bl/6 or B6D2F$_1$ mice than in CD-1 (germ-free) mice. Zaremba (105) was unable to establish LD_{50}s in Swiss mice and showed that nonirra-diated Balb/c mice were less susceptible to infection with Y. enteroco-litica than were irradiated mice. Quan et al. (77) showed that some strains of Y. enterocolitica, incapable of producing mortality in white mice, produced morbidity in these animals when they were challenged subcutaneously (sc). They also found one strain, CDC-C2417, extremely pathogenic for gerbils, killing each one within four days when chal-lenged ip with as few as 250 organisms.

Carter and Collins (26) investigated the kinetics of infection

in mice of the WA strain. They found that bacteria grown at 25 C
were more resistant to intracellular killing, and were cleared from
the blood at a slower rate, than were organisms grown at 37 C. Sev-
eral other investigators reported observing a similar temperature-
dependent effect. Lee et al. (63) found that some strains of Y. ente-
rocolitica were more invasive for HeLa cells in vitro when grown at
22 C than when grown at 36 C. However, they also found strains which
were invasive no matter what the growth temperature. Krynski et al.
(60) demonstrated that strains cultivated at 20 C were nearly 100%
lethal for lice when inoculated intrarectally into them. However,
when these strains were cultivated at 32 C, partial, or even complete,
recovery of the lice was observed. Pai and Mors (75) showed that the
production of heat-stabile enterotoxin by Y. enterocolitica was tem-
perature-dependent. It was produced by strains grown at 22-26 C,
but not at 37 C. Also, Sakazaki et al. (83) found enterotoxigenic
activity in cell-free filtrates of cultures grown at 30 C on ligated
gut loops from rabbits. In contrast, however, Quan et al. (77) were
unable to demonstrate the presence of any toxin(s) in cell-free ex-
tracts of Y. enterocolitica strains grown at 25 or 37 C. Also, Ric-
ciardi et al. (80) reported no temperature-dependent effect on long-
term fecal excretion of Y. enterocolitica in multiply infected Porton
white mice. They challenged mice ip with an inoculum of a serotype
0:3 or 0:9 strain grown at 25 or 37 C and then secondarily challenged
ip, several weeks later, with the WA strain. They found that the WA
strain was excreted in feces with the same frequency regardless of the
growth temperature of the initial inoculum. A study on temperature-

dependent production of endotoxin by this bacterium has not been done.

Associations between the source of isolates and their experimental pathogenic activity were studied. Carter et al. (27), Alonso et al. (5,7), and Bercovier et al. (14) showed only human isolates of Y. enterocolitica to be pathogenic for mice. Maki et al. (65) reported that 21 out of 24 clinical isolates of Y. enterocolitica were invasive for human epithelial (HEp-2) cells in vitro. Lee et al. (63) showed that biochemically typical strains of human origin were invasive for HeLa cells in vitro, while biochemically atypical, rhamnose fermenting strains of human origin and all strains of nonhuman origin were not invasive. In contrast, Une (98) demonstrated that at least one environmentally isolated strain of Y. enterocolitica was invasive for HeLa cells and rabbit peritoneal macrophages in vitro. Une (97) also reported that this isolate was pathogenic for rabbits challenged intraduodenally with it. All clinical isolates of Y. enterocolitica that he tested were also active in these assay systems (97,98). Quan et al. (77) showed that several nonhuman isolates were capable of producing morbidity or mortality in mice. Also, Pai et al. (76) found that clinical and nonclinical isolates of Y. enterocolitica were enterotoxin producers.

Although Mollaret and Guillon (70) reported finding no relationship between the O-serotypes of Y. enterocolitica and in vivo pathogenicity in laboratory animals, recently there were reports which indicate otherwise. Une (100) demonstrated that serotypes O:3, O:5B, O:8, and O:9, regardless of their source of isolation, were highly

invasive for HeLa cells in vitro. Maki et al. (65) showed that nearly
all of the 21 strains of Y. enterocolitica invasive for HEp-2 cells
in vitro were 0:3 or 0:9 serotypes. Pai et al. (76) observed that
human and nonhuman serotypes 0:3, 0:5,27, 0:6,30, 0:8, and 0:9 were
usually capable of enterotoxin production. However, they noted that
other serotypes, independent of source, also were enterotoxin produ-
cers. In addition, Sakazaki et al. (83) showed that serotype 0:3
culture filtrates were enterotoxigenic for rabbit ileal loops, but
that serotype 0:10 culture filtrates were not.

Some investigators postulated that toxin production may contri-
bute to the pathogenicity of Y. enterocolitica in humans (16,25,38,55,
62,75,83). Lee et al. (63) suggested that the rhamnose-positive
strains of human origin not invasive for HeLa cells might be entero-
toxigenic. However, Pai et al. (76) showed that, except occasionally,
rhamnose-positive isolates of clinical and nonclinical origin were
not enterotoxin producers. Isolation of endotoxin from the WA strain,
grown at 37 C, was accomplished by Carter (25). He found it less
potent, weight for weight, than a similarly extracted endotoxin from
Salmonella typhi. However, he acknowledged that this lower order
of potency might be due to low endotoxin content of the organism under
these growth conditions. Kanamori (55) reported on trichloroacetic
acid and phenol-water extracted endotoxins from Y. enterocolitica
0:3 and 0:9 serotypes. He found that their chemical compositions
and biological activities were similar to those of identically pre-
pared endotoxins from E. coli. Thus, he concluded that endotoxin may
play a role in human infections due to Y. enterocolitica. Gripenberg

et al. (38) suggested that an adjuvant effect exerted by endotoxin from Y. enterocolitica in combination with tissue antigens, released by a previously inflamed intestinal epithelium, could trigger an autoimmune response in the host. Such an effect might account for the cases of post-yersionitic arthritis and erythema nodosum observed with increasing frequency (3,16,38,74). Therefore, enterotoxin and/or endotoxin could be pathogenetic factors of Y. enterocolitica infection in humans.

This study was designed to further describe phenol-water extracts from strains of Y. enterocolitica selected for certain characteristics. A rhamnose-negative, human serotype O:8 and a rhamnose-positive, environmental serotype O:17 were chosen for cultivation at 25 and 37 C, followed by aqueous-phenol extraction for LPS. The chemical composition and biological activities of all fractions from each extraction were characterized by using various assay systems. These included tests for lipid, KDO, hexosamine, and carbohydrate content, and for lethal, pyrogenic, mitogenic, and tumor suppressive activities. These studies provided information on the nature of the LPS obtained from different strains grown at different incubation temperatures. Such characterization of the LPS may be pertinent to the taxonomic status of Y. enterocolitica and its relationship to the Enterobacteriaceae, and to the possible contribution of endotoxin to pathogenicity of the organism.

CHAPTER II

MATERIALS AND METHODS

Bacterial strains.

Two <u>Y. enterocolitica</u> strains were used in this study. They were obtained from Dr. T. J. Quan, Center for Disease Control, Ft. Collins, Colorado. One, #CDC-C2417, was identified as a rhamnose-negative, serotype O:8, human spleen isolate, which was pathogenic for mice and gerbils (39,77). The second, #TFW-CM6045x1, was identified as a rhamnose-positive, serotype O:17, water isolate, whose experimental pathogenicity was not known. These strains were maintained as stock cultures on tryptic soy agar (Difco Laboratories, Detroit, MI) slants kept at 4 C and transferred every 8 to 10 weeks.

Preparation of extracts.

Two different batches of cells were collected from each <u>Y. entero-colitica</u> strain for extraction. One batch was grown at 25 C and the other was grown at 37 C. Thus, a total of 4 batches of cells were prepared in all. Each batch was grown and harvested in the following manner. A loopful of organisms from the stock slant was inoculated to each of 4 one liter flasks containing 250 ml of brain heart infusion (BHI, Baltimore Biological Laboratory, Cockeysville, MD) broth which were then incubated at either 25 or 37 C for 24 hours on a shaker. After 24 hours, these starter cultures were added to 9 liters of BHI

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broth and this new growth suspension was incubated in a Microferm fermenter (New Brunswick Scientific, New Brunswick, NJ) at the same temperature as the starter cultures, with 300 rpm agitation and 4 liters/minute aeration at 8.5 pounds/square inch pressure. A 20% solution of Antifoam B (Sigma Chemical Co., St. Louis, MO) was added as needed. Growth was followed by optical density using a Coleman Model 6/35 spectrophotometer (Coleman Instruments, Maywood, IL) at a wavelength of 600 nm, using BHI broth as a medium blank. It also was followed by viable count with serial dilutions of aliquots of the growth medium in 0.85% saline inoculated to plate count agar (Difco Laboratories) plates in duplicate. The number of colonies were counted following incubation at 25 C for 24 hours. After 6 hours incubation in the fermenter, growth entered late logarithmic phase. The cells were harvested by transfer of the culture medium to 250 ml centrifuge bottles, followed by centrifugation at 3000 rpm and 4 C for 30 minutes in an International Model UV centrifuge (International Equipment Co., Needham Heights, MA). The culture medium was decanted and the cells were washed three times, twice by resuspension in 0.85% saline, and the third time by resuspension in distilled water. Each wash was followed by centrifugation at 3000 rpm and 4 C for 30 minutes. After the last wash, the cells were resuspended in a small volume of distilled water and lyophilized.

For the phenol-water extraction, the lyophilized cells were re-constituted in distilled water at a concentration of 100 g/liter. The bacteriological purity of each suspension was checked by direct gram stain and by comparison of its biochemical profile against that

of the parent stock culture using the API 20E identification system
(Analytab Products, Plainview, NY). In all instances, gram-negative
rods with a biochemical profile identical to that of the parent strain
were found. The extraction of the cell preparations was by a modifica-
tion of the aqueous-phenol method reported by Ribi et al. (79). This
procedure is summarized in Figure 1. Four fractions from each batch
of cells were collected: 1) A pooled cell debris phase, phenol phase,
interphase fraction, 2) An aqueous (crude LPS) fraction, 3) A sediment
(purified LPS) fraction from the ultracentrifugation of fraction 2,
and 4) A pooled supernatant fluid (cytoplasmic) fraction from the
ultracentrifugation of fraction 2. Since 4 different batches of cells
were harvested, the O:8 and O:17 strains each grown at 25 and 37 C,
a total of 16 distinct fractions were obtained. The % yield of each
fraction was determined by the following equation:

$$\% \text{ yield} = \frac{\text{dry weight of fraction}}{\text{dry weight of cells}} \times 100\%$$

The purification of fraction 2 by ultracentrifugation resulted
in a 50- to 100-fold decrease in RNA content of fraction 3. This loss
of RNA was determined by spectrophotometric scan of aliquots of material
suspended in distilled water over a wavelength range of 220 to 300 nm
with an Hitachi-Coleman Model 124 spectrophotometer (Hitachi-Ltd.,
Tokyo, Japan). A marked decrease in absorbance between wavelengths
of 245-260 nm of fraction 3 material as compared to fraction 2 mate-
rial was observed in these ultraviolet scans.

Ec 434, a LPS extract from E. coli 0113, was a gift of Dr. J. A.
Rudbach, Dept. of Microbiology, University of Montana, Missoula, Mon-
tana, and BLP 011, a lipoprotein extract of LPS from Salmonella minne-

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Step 1.

Lyophilized whole cells were suspended in distilled water at a concentration of 100 g/liter and warmed to 68 C.

An equal volume of 88% phenol (J. T. Baker Chemical Co., Phillipsburg, NJ), preheated to 68 C, was added to the cell suspension, and the mixture was kept at 68 C, with stirring, for 30 minutes. An homogenous suspension resulted.

The mixture was cooled to 4 C and centrifuged in an International refrigerated centrifuge, Model PR-6 (International Equipment Co., Needham Heights, MA), at 2500 rpm for 30 minutes. At this point, four distinct phases were observed.

The aqueous phase was aspirated off. The other three phases were pooled and, after addition of a volume of distilled water equal to that removed, were reextracted 2 more times as described above.

The inter-, phenol, and cell debris phases were pooled and dialyzed against distilled water at 4 C for three weeks and then lyophilized. This material was labeled fraction 1.	The three aqueous phases were pooled and centrifuged in the refrigerated centrifuge at 2500 rpm for 30 minutes to remove further debris. After decantation, they were dialyzed against distilled water at 4 C until all the phenol was removed and then lyophilized. This material was labeled fraction 2.

Step 2.

A portion of fraction 2 was resuspended at a concentration of 3%, weight to volume, in distilled water and was centrifuged at 80,000 g for 6 hours in a Beckman Model L2-65B ultracentrifuge (Beckman Instruments, Palo Alto, CA).

The supernatant fluid was decanted and saved. The sediment was resuspended in distilled water and centrifuged at 105,000 g for 3 hours.

The supernatant fluid was decanted and pooled with the first one. The pooled supernatant fluids were lyophilized and labeled fraction 4. The sediment was resuspended in a minimal amount of distilled water and lyophilized. This material was labeled fraction 3.

FIG. 1. Flow chart for the preparation of phenol-water extracts from strains of Y. enterocolitica by the method of Ribi et al. (79).

sota R595, was a gift of Dr. R. Wheat, Dept. of Microbiology, Duke University, Durham, North Carolina.

Assays for chemical composition.

Each of the 16 different extracts was assayed for its content of nitrogen, phosphorous, protein, fatty acid amides (FAA) and fatty acid esters (FAE), total carbohydrate, total hexose, hexosamine, and KDO. The amount of each chemical entity that was present in a sample of any one extract was determined quantitatively on an Hitachi-Coleman Model 124 (Hitachi-Ltd.) double beam spectrophotometer by comparison of sample absorptivities against those of suitably prepared standards, with one exception. Because a higher wavelength was required for the protein determinations, a Beckman DU-2 (Beckman Instruments, Fullerton, CA) single beam spectrophotometer was used instead for this assay. The following general formula was used to calculate the % content of each chemical species assayed for in this study:

$$\frac{\%\ \text{content}}{\text{of chemical}} = \frac{\text{Absorbance(sample)} \times \text{weight(standard)}}{\text{Absorbance(standard)} \times \text{weight(sample)}} \times 100\% \quad (1)$$

These values were determined at least in duplicate, in separate trials, for every chemical substance for each of the Y. enterocolitica extracts. However, slight modifications in equation 1 were required for several of the assays. These modifications will be submitted with the discussion of the affected assay.

The nitrogen content was determined by a modification of the Nessler reaction (54). This method, which involves a reaction of free nitrogen as ammonia, liberated by acid digestion of the sample, with mercuric iodide, produces a brownish colloidal suspension of $NHg_2I \cdot 2H_2O$.

The % nitrogen composition was calculated as per equation 1 after measurement of the absorbance, at a wavelength of 490 nm, of test and nitrogen standard solutions. A 1.0 mg sample in 0.5 ml of distilled water was used in the test solutions. Nitrogen standard solutions contained 0.3 ml of a 100 ug/ml stock solution of ammonium sulfate $((NH_4)_2SO_4$, J. T. Baker Chemical Co.) in distilled water.

The determination of phosphorous content was by a modification of the method reported by Dryer et al. (34). Instead of 1.0 ml of a 0.025 M solution of molybdate color reagent, as they suggested, 0.2 ml of a 0.008 M solution was used. The 0.008 M molybdate color reagent solution was prepared by a 1:3 dilution in distilled water of a stock solution containing 3.09 g of ammonium molybdate tetrahydrate $((NH_4)_6Mo_7O_{24} \cdot 4H_2O$, J. T. Baker Chemical Co.) dissolved in 100 ml of distilled water. The method ultimately depends upon the reduction of phosphomolybdic acid, formed by the reaction of ammonium molybdate with inorganic phosphate released by acid digestion of the sample, with development of a complex blue pigment which is presumably a mixture of molybdenum oxides. This reduction has been referred to as the "molybdenum blue" reaction. The absorbance of the test or the phosphate standard solution was read at a wavelength of 345 nm and the % phosphorous of the sample was calculated as per equation 1. Test solutions consisted of 0.5 mg of sample dissolved in 0.5 ml of distilled water. The phosphate standard was 0.5 ml of a 1:100 dilution in distilled water of a 1 mg/ml solution of monopotassium phosphate $(KH_2PO_4$, J. T. Baker Chemical Co.) in 5 N sulfuric acid.

The protein content was determined by Lowry's method as reported

by Herbert et al. (45). This method depends upon the "molybdenum blue" reaction and the copper complexing of amino acids for the development of spectrophotometrically determinable color. The absorbance of the test or the protein standard solution was read at a wavelength of 700 nm. However, instead of preparing a protein standard with each experimental trial, a protein standard curve, using samples of bovine serum albumin (Sigma Chemical Co., St. Louis, MO) ranging from 10 to 100 ug, was prepared for each set of reagents. This standard curve is given in Figure 2. Weighed samples of 100 ug were used for this determination and the % protein content of them was calculated with the following formula:

$$\frac{\% \text{ protein}}{\text{content}} = \frac{\text{Absorbance(sample)}}{\text{weight(sample)}} \times \frac{\text{slope of}}{\text{standard curve}} \times 100\%$$

Determination of the FAA plus FAE content was performed by the method of Haskins (44). This method depends upon the reaction of fatty acid esters and amides with alkaline hydroxylamine to form hydroxamic acids. These, in turn, will react with acid ferric perchlorate to give a purple iron chelate complex. The absorbance of these solutions was read at a wavelength of 530 nm. Instead of 5-10 mg of material, as suggested by Haskins (44), 2-3 mg of sample or 1-2 mg of standard was used. The FAA & FAE content was reported as % palmitate, using glycerol tripalmitate (Sigma Chemical Co.) as the standard. Calculation of this content was done by multiplication of equation 1 by 0.953, a factor which represented the percentage of palmitic acid in tripalmitin.

The total carbohydrate content was determined by the method of Dische (33). In this method, all carbohydrates, except hexosamines,

FIG. 2.   Protein standard curve prepared with 10, 50, and 100 ug samples of bovine serum albumin.   The slope of the curve is 0.2273 mg/absorbance unit.
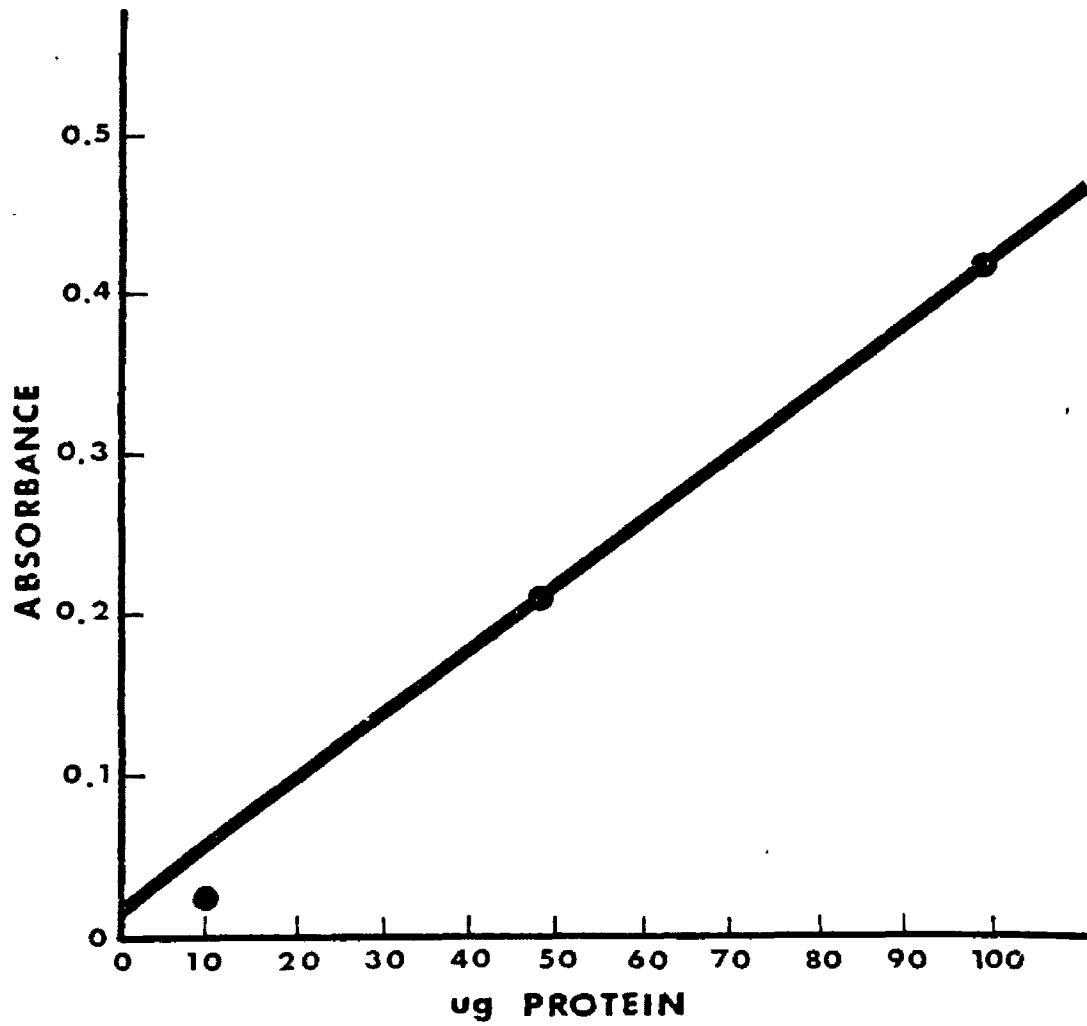
will react with tryptophan to form a brownish-red to violet-brown conjugation product. The % carbohydrate composition of the sample was calculated as per equation 1 after measurement of the absorbance, at a wavelength of 480 nm, of sample and carbohydrate standard solutions. Test solutions consisted of 100 ug of sample dissolved in 0.5 ml of distilled water. Duplicate standards of 50 ug of glucose (J. T. Baker Chemical Co.) dissolved in 0.5 ml of distilled water were run with each series of sample determinations.

Total hexose was assayed by a modification of the anthrone method as described by Scott and Melvin (89). This method depends upon conversion of hexoses to furfurals which then condense with anthrone to form colored products. The absorbance of the test or the hexose standard solution was read at 530 nm and the % total hexose composition was calculated by use of equation 1. The test solutions were prepared by dissolving 100 ug of sample in 1.0 ml of distilled water. Duplicate standards, each containing 30 ug of glucose (J. T. Baker Chemical Co.) dissolved in 1.0 ml of distilled water, were prepared with each series of sample determinations.

Determination of the hexosamine content was performed by a modification of Elson-Morgan's method (81). However, a further modification in this method was required in steps for the liberation of bound hexosamine. First, 1.0 ml of 3 N hydrochloric acid was added to 1.0 mg of sample, and the resulting solution was kept at 100 C for 6 hours. At the completion of hydrolysis, the solution was evacuated on an Evapo-Mix evaporator (Buchler Instruments, Ft. Lee, NJ) with agitation and heat. The resulting residue then was resuspended in 0.5

ml of distilled water. At this point, the remaining steps were per-
formed as per Rimington's modification (81). The Elson-Morgan method
involves a two-step reaction sequence. First, the hexosamine condenses
with acetylacetone (Sigma Chemical Co.) to give three or four chromo-
genic compounds. These compounds then react with Erlich's reagent
(p-dimethylaminobenzaldehyde, Sigma Chemical Co.) to form chromogens
whose absorbance can be determined at 530 nm. The absorbances of
weighed samples and glucosamine standards were read at this wave-
length and the % hexosamine was calculated by equation 1. Duplicate
tubes containing 0.25 ml of a 1:10 dilution, prepared in distilled
water, of a 1 mg/ml solution of glucosamine (Sigma Chemical Co.) in
distilled water were used for each series of sample determinations
as standards.

The KDO content was determined by a method described by Anacker
et al. (8). In this method, sugars with a configuration similar to
that of KDO are converted by periodate oxidation to malonaldehyde,
which then reacts with 2-thiobarbituric acid to form a violet colored
complex. The absorptivity due to the reaction of DDHs in this method
was adjusted for by measurement of absorbance at a wavelength of
550 nm before and after the addition of alkali. Chromogenic activity
due to the presence of KDO, and, presumably, other sugars, was de-
stroyed by heating in a boiling water bath with alkali, but color
due to the presence of DDHs was retained (8). Calculation of the
% KDO was accomplished by subtraction of the absorbance after alkali
treatment, multiplied by a factor of 0.398, from the absorbance before
alkali treatment. Since the absorption maximum of DDHs underwent a

wavelength shift after alkali treatment, the same concentration of
DDHs gave a higher absorbance reading after such treatment than they
did before such treatment. Thus, multiplication of the absorbance
after alkali treatment by 0.398, which represented the ratio of the
absorbance of DDH chromogen at 550 nm before alkali treatment to that
after alkali treatment, compensated for this effect. The adjusted
absorbances of test solutions and KDO standard solutions (no adjust-
ment for DDH content required) were used to calculate the % KDO com-
position of the samples by equation 1. Test solutions consisted of
100 ug of sample dissolved in 0.1 ml of distilled water. Standard
solutions consisted of 0.1 ml of a 100 ug/ml solution of KDO (2-keto-
3-deoxyoctonate, Sigma Chemical Co.) in distilled water.

Mouse lethality assay.

Toxicity of the various fractions was tested in mice. Suitably
prepared dilutions in distilled water of each fraction were injected
ip into white mice (Rocky Mountain Laboratory, Hamilton, MT) immediately
following potentiation with 10 ug Actinomycin D (Cosmegen, Merck, Sharp,
and Dome, West Point, PA) also given ip. Doses of the potentiator
and the test materials were contained in 0.2 ml volumes. Five mice
were used for each dose of material tested. The number of deaths
were recorded after 48 hours, and the amount of material, in ug, re-
quired to kill 50% of the mice (MLD_{50}) was calculated by the method
of Reed and Muench (78). No deaths were observed in control mice
receiving only the Actinomycin D.

The number of MLD_{50}s per 1.0 mg dry weight of cells was calculated

with the following formula:

$$\# \mathrm{MLD}_{50}\mathrm{s} = \frac{1.0 \text{ mg} \times \% \text{ yield of fraction}}{\mathrm{MLD}_{50} \text{ of fraction}} \quad (2)$$

Chick embryo lethality assay.[1]

Toxicity of the various extracts for chick embryos was determined as described by Milner and Finkelstein (69). Dilutions of test materials were prepared in pyrogen-free 0.15 M saline and 0.1 ml of each test dilution was injected iv into each embryo. Ten embryos were used for each dilution. Deaths were recorded at 24 hours and the amount of material, in ug, required to kill 50% of the chick embryos (CELD_{50}) was calculated by the method of Reed and Muench (78). There were no deaths in control embryos receiving only 0.1 ml of saline. Also, deaths due to post-inoculation hemorrhage were excluded from the data used in CELD_{50} determinations.

The number of CELD_{50}s per 1.0 mg dry weight of cells was calculated with a formula analogous to equation 2.

Pyrogenicity assay.[1]

Pyrogenicity assays in rabbits were done as described by Milner and Finkelstein (69). New Zealand White rabbits, 2 to 3 kilograms in weight and matched for age and sex, preconditioned to restraint in metal stanchions, were inoculated via the marginal ear vein with graded doses contained in 2.0 ml volumes. Temperatures were taken rectally

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[1]This assay was kindly performed by Dr. K. B. Von Eschen's laboratory, Rock Mountain Laboratory, Hamilton, Montana on preparations supplied to him by the author. A special note of thanks to Bob Pfeiffer who actually performed the assay.

with thermocouples and recorded by a calibrated telethermometer and recorder. Four animals were included at each test dose. Pyrogenicity was reported as the fever index forty (FI_{40}), or that dose in ug which gave a fever index of 40 centimeters squared. The fever index is the area under the fever curve in centimeters squared where 1 hour and 1 C are plotted as 1 inch.

The number of FI_{40}s per 1.0 mg dry weight of cells also was calculated with a formula analogous to equation 2.

Mitogenicity assay for T- and B-lymphocytes.

Mitogenicity assays were done in C3HeB/FeJ and C3H/HeJ (Jackson Laboratories, Bar Harbor, ME), and nude (Rocky Mountain Laboratory) mice by the following method. Spleens were aseptically removed from the mice into sterile phosphate-buffered balanced salts (PBBS) and separated into single cell suspensions by grinding them between sterile ground glass slides. The cells were washed twice with PBBS and finally resuspended in RPMI-1640 medium (Microbiological Associates, Bethesda, MD) supplemented with 5% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 ug/ml) at a concentration of 1 x 10^6 viable cells/ml. Then 0.2 ml of medium, containing 2 x 10^5 cells, was added to individual wells of a microtiter plate (IS-FB-96TC, Limbro Scientific, Inc., Hamden, CT). Different doses of the test fractions were added to the wells, and the cultures were incubated for 24 hours in a humidified incubator at 37 C in an atmosphere of 5% CO_2 in air. After incubation, the cultures were pulsed with 1 microCurie of ^{3}H-methyl-thymidine (^{3}HTdr; 6.7 curies/millimole, New England Nuclear)

and returned to the incubator for an additional 24 hours. Next, they were harvested on filter pads, with repeated washings with saline and distilled water, using an automated multiple harvester (Otto Hiller, Inc., Madison, WI). The individual filter discs were placed into miniscintillation vials and allowed to dry in a 37 C incubator. The number of radioactive counts were tallied in a Beckman LS 8100 (Beckman Instruments, Fullerton, CA) liquid scintillation counter after each vial had received 1.0 ml of scintillation fluid. All cultures were done in triplicate.

The data was expressed as the stimulation index, which was the ratio of the average counts per minute of the experimental cultures over the average counts per minute of the background control cultures. Background control cultures received only cells. Dilutions of Con-canavalin A (Con A), Ec 434, and BLP 011 were included in each series of determinations as positive controls. Con A was shown to be a T-cell mitogen and Ec 434 and BLP 011 were shown to be B-cell mitogens (102).

Tumor suppression assay.

Fibrosarcoma tumor cells (MC-93), induced in a C3H mouse by 3-methylcholanthrene, were obtained from Dr. K. B. Von Eschen, Rocky Mountain Laboratory, Hamilton, Montana. These cells were maintained in vitro in tissue culture medium (Minimum Essential Medium, Eagle, Microbiological Associates) supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 ug/ml), and 15 mM Hepes (Sigma Chemical Co.). The cells were subcultured into fresh medium

every 7-10 days.

For the tumor suppression assay, MC-93 cells were harvested from tissue culture flasks, washed twice in PBBS, and resuspended in PBBS at a final concentration of 2×10^6 viable cells/ml. Suitable dilutions of the materials to be tested were prepared in PBBS. Next, test and tumor cell suspensions were mixed together in equal volumes, and, immediately, 0.1 ml of the mixture was injected sc into each mouse. Control mice received 0.1 ml of a suspension prepared with equal volumes of tumor cells and PBBS. Mice receiving injections included C3HeB/FeJ and C3H/HeJ strains, LPS responders and nonresponders, respectively (Jackson Laboratories). Ten mice were used for each test mixture. They were examined post-inoculation once a week for six weeks for detectable tumors at the site of injection. Data was recorded as the cumulative incidence of tumors at the conclusion of the experiment. The tumor incidence in control mice of both strains was 100%.

Statistics.

Statistical differences between data were determined by Student's t-test. Means and standard deviations of multiple determinations of assays were calculated. For any two means, $\overline{X}_1$ and $\overline{X}_2$, from independent data sets 1 and 2 of the same chemical or biological assay, a z-value was calculated with the following formula:

$$z = \frac{\overline{X}_1 - \overline{X}_2}{(o_1^2/n_1 + o_2^2/n_2)^{1/2}}$$

where $\overline{X}$ = mean, o = standard deviation, and n = sample size and sub-

CHAPTER III

RESULTS

The primary goal of this study was to examine possible strain
dependent or temperature-dependent variations in production, composi-
tion, or activity of LPS extracted from Y. enterocolitica. Therefore,
it was first necessary to identify which, if any, fraction(s) of the
extraction procedure contained LPS. In the phenol-water extraction
of other bacteria, LPS normally was obtained in the aqueous phase,
and purification by ultracentrifugation resulted in sedimentation of
the LPS (4,79). When calculated, the % yield of sedimentable material
from the dry weight of whole cells generally was less than 5%, but was
variable (4). Table 1 contains data on the % yields of all the frac-
tions obtained in this study. It can be seen that the % yield of frac-
tion 3 extracts, the sediments from ultracentrifugation, were consis-
tent with the above criterion; only the fraction 3 extract from the
0:17 strain grown at 37 C demonstrated a very low % yield. It also
can be seen that a strain dependent and temperature-dependent variation
in production of fraction 2 and 3 material occurred. Twice as much
fraction 2 material and four times as much fraction 3 material was ob-
tained from cells cultivated at 25 C than at 37 C for each strain.
In addition, approximately four times as much fraction 3 material
was obtained from the 0:8 strain than from the 0:17 strain at each
growth temperature. However, since % yield of sedimentable material

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TABLE 1. Percent yield of extracts from the dry weight of whole cells of $\underline{Y.}$ $\underline{enterocolitica}$ O:8 and O:17 strains grown at different temperatures.

Bacterial strain	Growth temperature[a]	Fraction			
		#1	#2	#3	#4
O:8	25	55.21%	28.31%	5.39%	20.26%
	37	46.00%	13.39%	0.98%	10.10%
O:17	25	59.54%	20.28%	1.37%	16.02%
	37	50.65%	8.74%	0.33%	6.98%

[a]In degrees centigrade

was not an invariant property of·LPS, examination of the various
fractions for a more specific LPS marker was required.

KDO was shown to be an unique sugar characteristic of the R-core
region of the LPS from virtually all of the Enterobacteriaceae (4,42,
84). Therefore, examination of the fractions for KDO content was
undertaken. Table 2 presents the information obtained. From cells
grown at 25 C the greatest KDO content was found in fraction 3 mate-
rials. However, at 37 C, the greater part of it remained in the
supernatant fluid, fraction 4, after ultracentrifugation. Although
the % KDO composition of 37 C fraction 3 extracts were less than those
from the corresponding 25 C extracts, they still probably reflected
significant yields of LPS in these fractions. These temperature-depen-
dent differences in KDO composition of the fraction 3 and 4 extracts
of both strains were significant at the p less than 0.05 level or
better. The % KDO composition of fraction 2 material from the 0:17
strain displayed a reverse temperature-dependent phenomenon. More
KDO was obtained from 37 C than from 25 C cultured cells for this
strain. This difference was significant at the p less than 0.01 le-
vel. Not shown, but observed in this assay, was a significant content
of DDH(s) in the 0:17 strain but not in the 0:8 strain. However, the
amount of this material present was not determined. The presence of
measurable KDO was not sufficient to classify an extract as LPS, since
the assay for KDO was nonspecific, occurring with any sugar possessing
a configuration similar to that of the 2-keto-3-deoxy group of KDO.
Indeed, the KDO contents of fraction 1 extracts were so slight as to
be considered nonspecific. Therefore, the fractions were tested for

TABLE 2. Temperature-dependent KDO composition of Y. enterocolitica O:8 and O:17 extracts.

Extract fraction	% KDO[a]		
	25 C	37 C	p[b]
O:8 strain			
#1	0.41	0.46	>0.05
#2	4.19	3.75	>0.05
#3	5.53	1.46	<0.01
#4	0.76	6.08	<0.05
O:17 strain			
#1	0.59	0.40	>0.05
#2	1.80	5.32	<0.01
#3	3.01	0.81	<0.05
#4	1.34	5.10	<0.05

[a]All values reported as averages of % composition from 2 or more independent trials.

[b]Level of significance of observed difference between the two reported values.

the presence of other chemical substances.

Analyses for the % composition of nitrogen, phosphorous, protein, lipid, and carbohydrates of all the fractions were accomplished. These data for fraction 3 extracts from both of the Y. enterocolitica strains are given in Table 3. Essentially, the chemical compositions within and between the strains were alike. However, two differences of values reported in this table were significant, one within the O:8 strain, and the other between the strains. First, LPS from cells of the O:8 strain grown at 25 C contained less than 1% protein, while that from cells grown at 37 C contained 7.14% protein. This difference was significant at the p less than 0.05 level. In the second case, the total carbohydrate content of LPS from the O:17 strain was much greater than that from the O:8 strain, regardless of the growth temperature. This difference also was significant at the p less than 0.05 level. Compositions of fraction 2 and 4 extracts were similar to those of corresponding fraction 3 extracts, differing primarily in KDO content as demonstrated in Table 2. Also, the fraction 4 extracts contained a greater phosphorous content, on the order of about 5%. Since a decrease in the RNA concentration of the fraction 3 extracts after ultracentrifugation was observed in ultraviolet scans, the larger phosphorous content in fraction 4 probably reflected the retention of nucleic acids in this material. Fraction 1 extracts from both strains uniformly contained approximately 50% protein and 30% lipid with, usually, less than 5% carbohydrate, regardless of the temperature of incubation. In as much as growth temperature of cells used for extraction demonstrated little variation in chemical composition of the extracts, the 25 C

TABLE 3. Chemical composition of fraction 3 extracts from _Y. entero-colitica_ O:8 and O:17 strains grown at 25 and 37 C.

Bacterial strain	O:8		O:17	
Growth temperature[a]	25	37	25	37
% Total[b]				
Nitrogen	1.53	0.77	2.04	0.75
Phosphorous	1.52	0.48	1.36	0.90
Protein	<1.00	7.14	3.57	4.76
FAA & FAE	27.7	31.1	29.5	23.5
Carbohydrate	49.6	37.9	68.3	62.9
Hexose	27.7	27.4	32.3	44.4
Hexosamine	17.0	14.5	8.0	17.0
KDO	5.53	1.46	3.01	0.81

[a]In degrees centigrade.

[b]All values reported as average % chemical content from 2 or more independent trials.

TABLE 4. Chemical composition of LPS phenol-water extracts from Y. enterocolitica strains grown at 25 C compared to that of E. coli 0113 LPS.

% Total[a]	E. coli 0113[b]	O:8 strain	O:17 strain
Nitrogen	2.60	1.53	2.04
Phosphorous	1.35	1.52	1.36
Protein	NA[c]	<1.00	3.57
FAA & FAE	35.0	27.7	29.5
Carbohydrate	34.4	49.6	68.3
Hexose	26.2	27.7	32.3
Hexosamine	16.4	17.0	8.0
KDO	3.88	5.53	3.01

[a]All values reported as an average % composition

[b]After Rudbach et al. (82).

[c]NA = not available.

fraction 3 materials are compared in Table 4 to the composition of LPS from E. coli, obtained by similar methods, as reported by Rudbach et al. (82). It can be seen that the fraction 3 extracts generally resembled LPS from E. coli in their chemical composition. Because of this strong chemical equivalency, the examination of the biological activities of fraction 3 materials were pursued to provide further corroboration of their identification as LPS.

The next experiments analyzed the capacity of extracts from Y. enterocolitica to be toxic for white mice and 11 day old chick embryos, and pyrogenic for rabbits. These last two assays were standard tests used to determine endotoxicity of test reagents (69,102). Data from these assays on each of the 16 fractions are given in Tables 5 and 6. The fever response to varying doses of fraction 3 extracts is given in Figure 3. Not surprisingly, a high level of potency was found in the fraction 2, 3, and 4 extracts, but not in the fraction 1 materials. Fraction 3 presumably was the endotoxin containing fraction. The fraction 2 material was a crude LPS preparation from which fraction 3 was purified by ultracentrifugation. The relatively high biological potency of the fraction 4 extracts probably reflected contamination with LPS but it was also possible that these materials contained some additional toxic factor(s). Closer examination of the biological activities of the fraction 3 preparations revealed two points. First, with one apparent exception, within each strain a temperature-dependent variation in biological activities of fraction 3 materials was not observed. At first, examination of the FI_{40}s of fraction 2 and 3 materials appeared to demonstrate a temperature-dependent difference.

FIG. 3. Dose response curves of fever in rabbits produced by fraction 3 extracts from Y. enterocolitica O:8 and O:17 strains. Each point is an average of the fever indices of 4 rabbits, where the fever index is the area under the fever curve. The temperatures indicate the growth temperatures of the cultures prior to extraction.
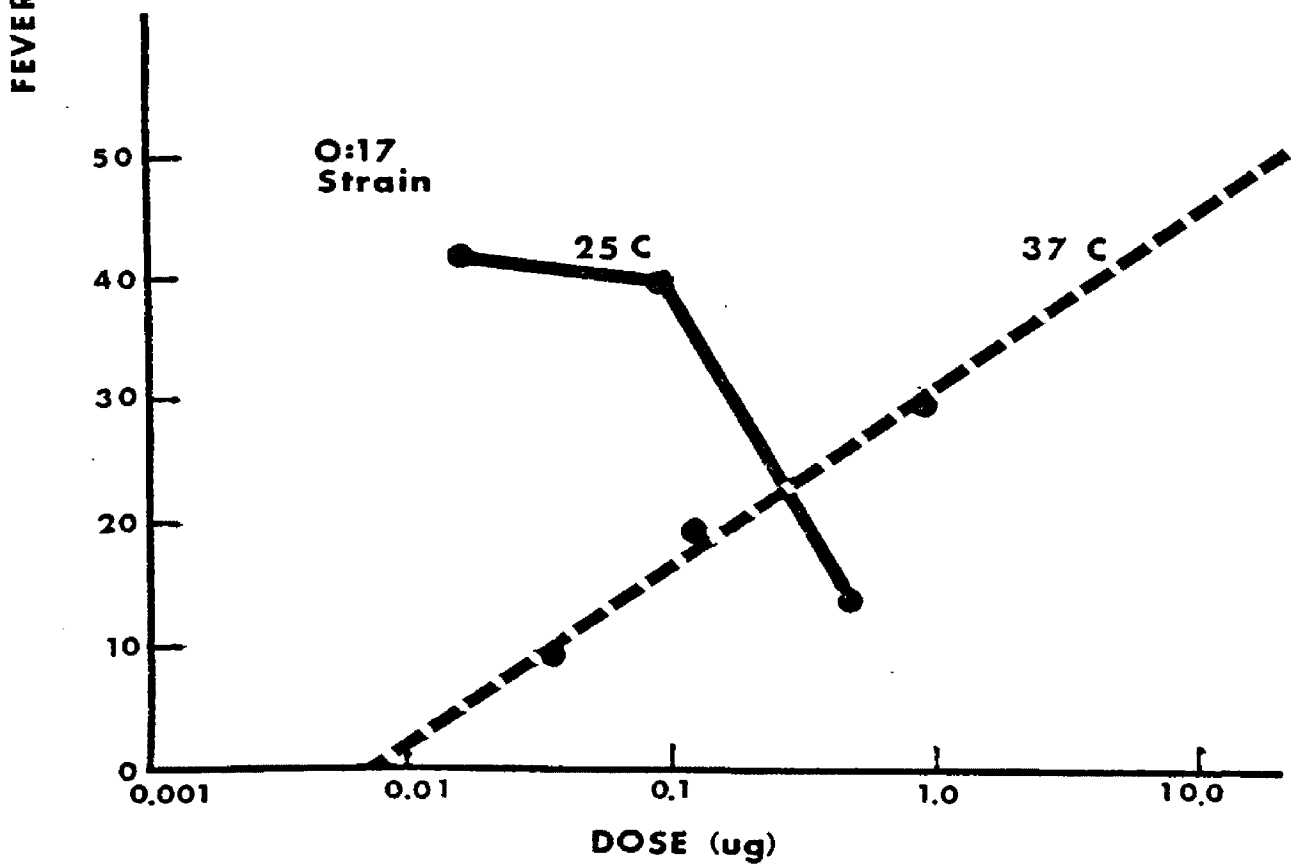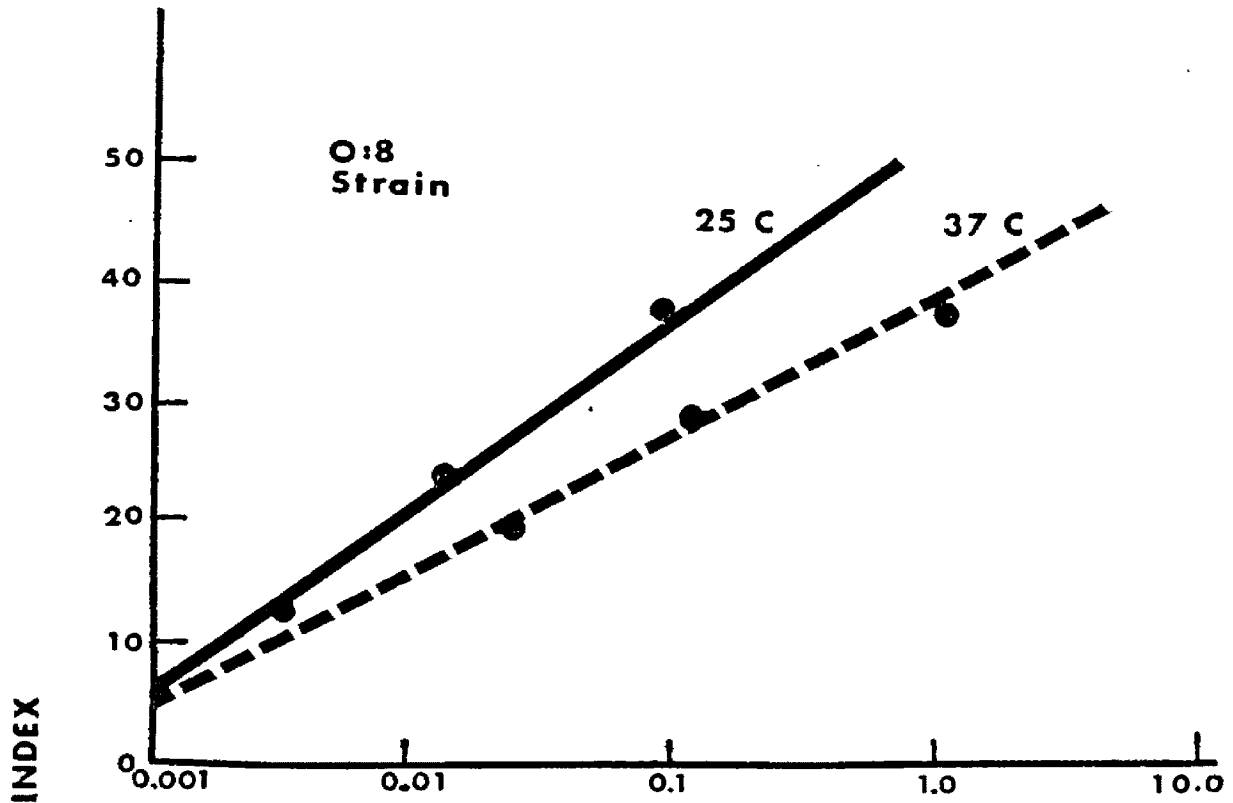
However, repeated trials of fraction 2 materials in this assay system revealed no significant temperature-dependent difference in pyrogenic activity. More specifically, the average FI_{40} for fraction 2 material from the O:8 strain at 25 C was 0.22 ug and at 37 C was 0.85 ug. The difference between these values was not significant at p less than 0.05. The same was true of the O:17 strain where the average FI_{40} of fraction 2 extract at 25 C was 0.066 ug and at 37 C was 0.14 ug. The difference in these values also was not significant at p less than 0.05. Presumably, this lack of significance in the FI_{40}s extended to the fraction 3 materials as well. Second, no difference in absolute biological activity of fraction 3 extracts between the strains was apparent. However, strain dependent differences in the biological activities of fractions 1 and 4 were found. Comparison of the MLD_{50}s, $CELD_{50}$s, and FI_{40}s of these materials indicated that the O:17 strain possessed more potent preparations. The differences in activity were more obvious for 25 C extracts than for 37 C ones, especially for fraction 1 materials. For example, from Table 5 the fraction 1 extract from the O:8 strain grown at 25 C displayed a MLD_{50} of 5.98 ug, a $CELD_{50}$ greater than 20 ug and a FI_{40} greater than 100 ug. From Table 6, fraction 1 material from the O:17 strain matched for temperature exhibited a MLD_{50} of 0.23 ug, a $CELD_{50}$ of 0.2 ug, and a FI_{40} of 12.9 ug, approximately a 10-fold difference, or better, in potency from the O:8 strain. Comparison of the values in Tables 5 and 6 will show that the same relationship was true of the fraction 4 extracts as well. At this point, the evidence accumulated strongly supported labelling fraction 3 materials from Y. enterocolitica as LPS. However, additional bio-

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logical characterization of these substances would substantially strengthen such identification.

LPS was found to demonstrate different biological activity in closely related strains of animals depending upon their genetic capacity to respond to LPS (4,102). For instance, LPS was shown to be a potent mitogen for B-lymphocytes in C3HeB/FeJ mice but not in the related C3H/HeJ strain (102). Thus, spleen cells from these strains cultured *in vitro* were stimulated to divide or not divide, respectively, in the presence of LPS. Therefore, the mitogenic activity of the fraction 3 preparations for spleen cells from C3HeB/FeJ and C3H/HeJ mice was tested. In addition, mitogenic activity for spleen cells from nude mice also was determined. The results were tabulated as the stimulation indices and are reported in Figures 4 and 5 and Table 7. Determinations of the mitogenic activities of known substances were also included in these experiments. These included a LPS extract of *E. coli*, Ec 434, a B-cell mitogen, Con A, a T-cell mitogen, and BLP 011, another B-cell mitogen. In general, as shown in Figures 4 and 5, spleen cells from C3HeB/FeJ mice responded mitogenically, but those from C3H/HeJ mice did not. One exception to this observation was the mitogenic response of C3H/HeJ spleen cells to fraction 3 extract from the O:17 strain grown at 37 C. Table 7 demonstrated that the responses of the fraction 3 extracts, at a specified dose, were very similar to those of Ec 434.

The mitogenic profiles of fractions 1, 2, and 4 were also determined. Mitogenic activities in fraction 2 and 4 extracts were similar to the corresponding fraction 3 extracts in both strains, indicating,

FIG. 4. Mitogenic activity for murine spleen cells of fraction 3 extracts from Y. enterocolitica O:8 and O:17 strains grown at 25 C. Each point is an average of three determinations of radioactivity, after uptake of tritiated thymidine, reported as the stimulation index. C3HeB/FeJ and C3H/HeJ mouse strains, LPS responders and nonresponders, respectively, were used.
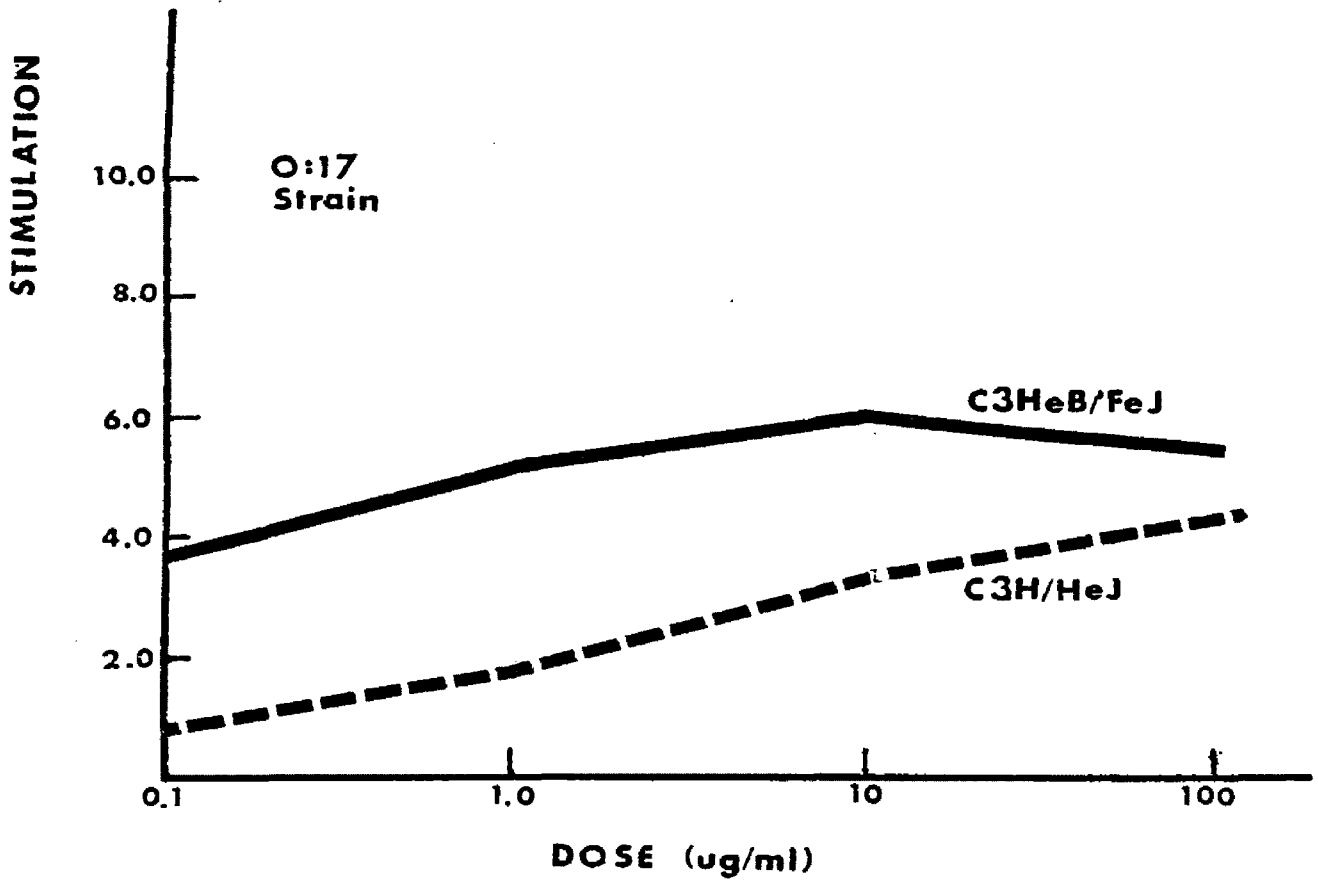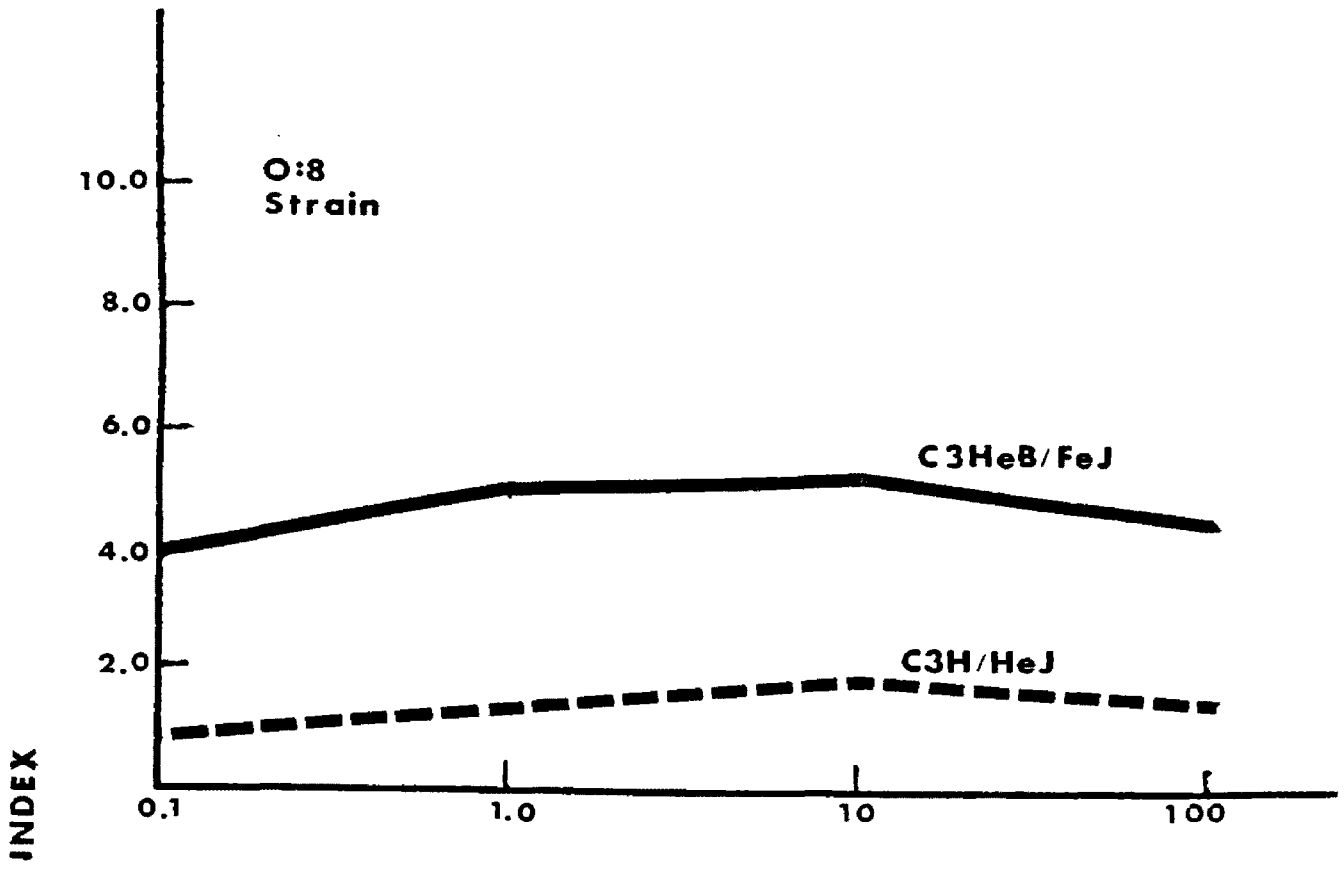
FIG. 5. Mitogenic activity for murine spleen cells of frac-
tion 3 extracts from Y. enterocolitica O:8 and O:17 strains grown
at 37 C. Each point is an average of three determinations of radio-
activity, after uptake of tritiated thymidine, reported as the
stimulation index. C3HeB/FeJ and C3H/HeJ mouse strains, LPS respon-
ders and nonresponders, respectively, were used.
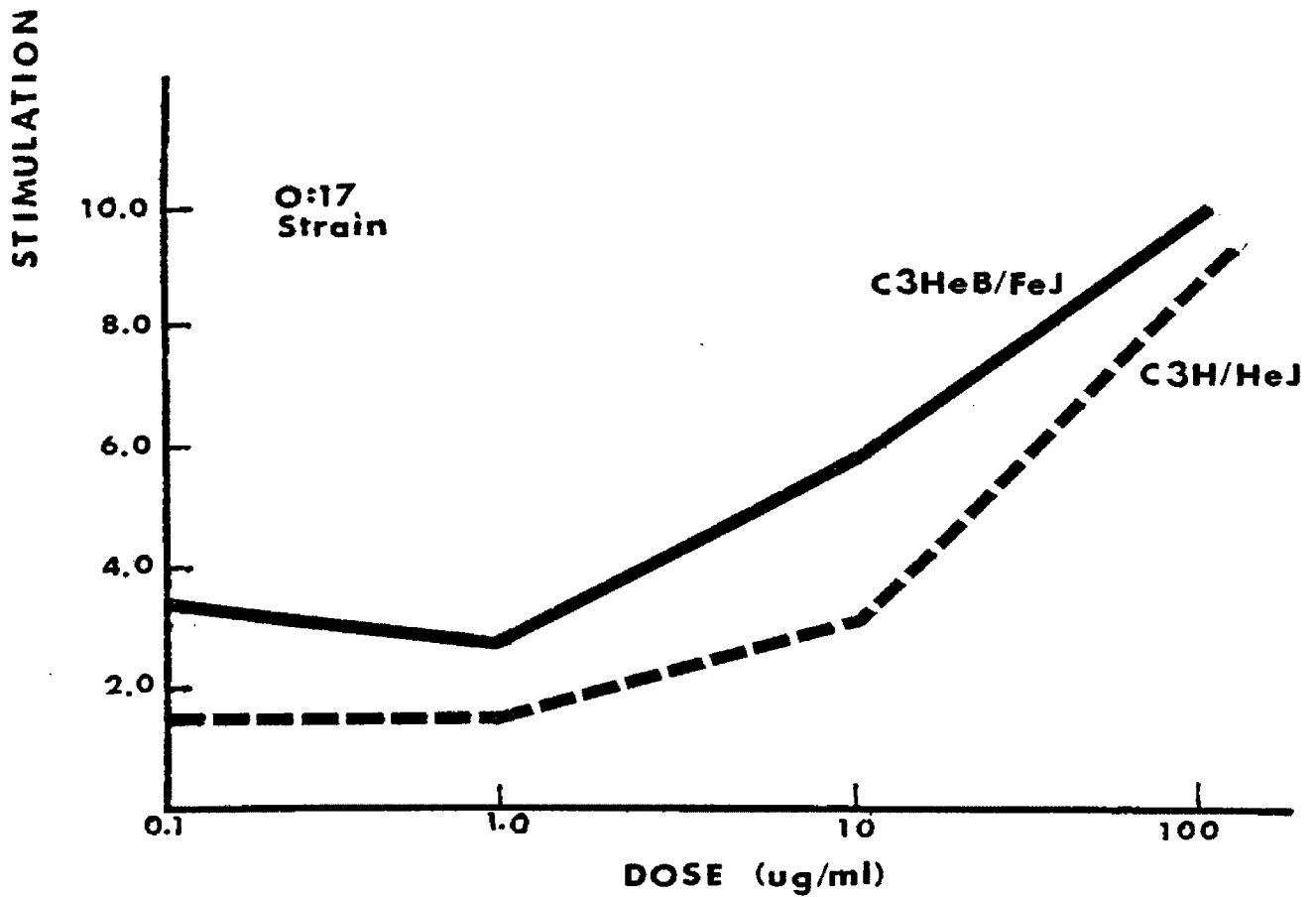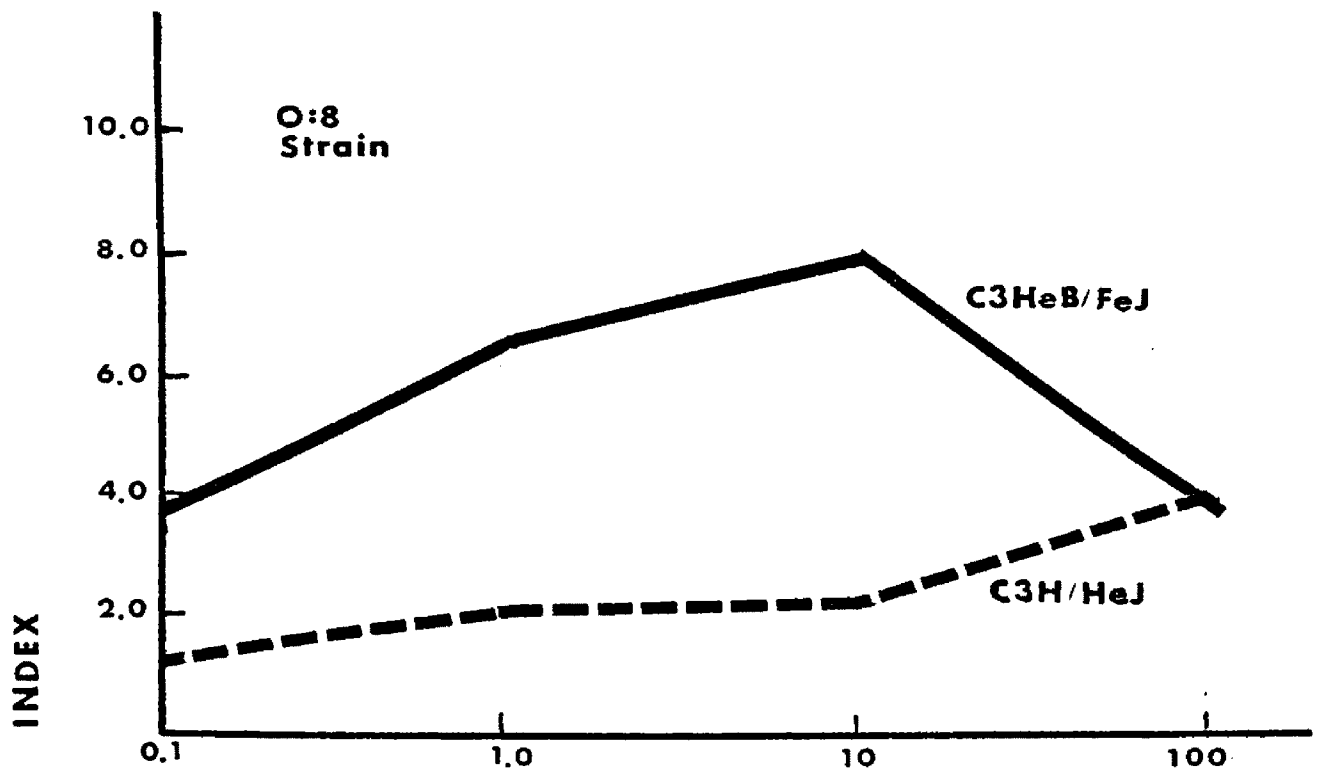
TABLE 7. Comparison of the mitogenic activities[a] of LPS from _Y. enterocolitica_ strains against those of control extracts.

Extract[b]	Growth temperature[c]	Mouse Strains C3HeB/FeJ[d]	C3H/HeJ[d]	nude[e]
0:8 LPS	25	5.21	1.69	3.61
	37	7.72	2.57	6.18
0:17 LPS	25	5.95	3.39	3.89
	37	5.78	3.45	3.46
Ec 434[f]	NR[g]	6.28	1.73	5.46
Con A[h]	NR[g]	2.29	6.48	0.99
BLP 011[i]	NR[g]	5.20	5.76	4.38

[a]Reported as an average stimulation index.

[b]All extracts at doses of 10 ug/ml, except Con A which was 1 ug/ml.

[c]In degrees centigrade.

[d]Jackson Laboratories, Bar Harbor, ME.

[e]Rocky Mountain Laboratory, Hamilton, MT.

[f]LPS extract from _E. coli_ 0113.

[g]NR = not reported.

[h]Concanavalin A.

[i]Lipoprotein extract of LPS from _S. minnesota_ R595.

again, probable LPS content in these fractions. The mitogenic activities of the fraction 1 extracts were unlike any of the control substances tested, markedly stimulating spleen cells from each of the mouse strains. The pattern of low mitogenic activity of the fraction 3 materials in LPS nonresponding mice and high activity in LPS responding and nude mice provided further authentication of these substances as LPS.

Another aspect of variation of response to LPS in the above mouse strains was shown in tumor suppression activity. In the C3HeB/FeJ strain, injection of a mixture of tumor cells and LPS elicited a host response which prevented development of a tumor (102). Mice of this same strain receiving only tumor cells developed tumors. However, in C3H/HeJ mice, injection of tumor cells admixed with LPS demonstrated no antitumor activity (102). This response was shown to be dose dependent, with doses eliciting maximum suppressive activity approaching the minimum lethal dose (102). Investigation of the crude LPS preparations, the fraction 2 extracts, in this assay system was done. The data are given in Figure 6. With the exception of the 37 C fraction 2 material from the O:8 strain, suppression of tumors was observed in LPS responding mice, but not in nonresponding mice, for each of the crude LPS preparations. In fraction 2 material from the O:8 strain grown at 37 C 100% tumor incidence occurred in both mouse strains. There was 100% incidence of tumors in control mice of both strains receiving only tumor cells. Thus, tumor suppression activity of LPS from Y. enterocolitica in LPS responding mice was found.

A summary of the biological activities of fraction 3 extracts,

FIG. 6. Tumor suppression activity of fraction 2 extracts from Y. enterocolitica O:8 and O:17 strains. C3HeB/FeJ and C3H/HeJ mouse strains, LPS responders and nonresponders, respectively, were used. Each mouse received a dose of 250 ug of material admixed with 1×10^5 tumor cells. Control mice received only tumor cells. The temperatures indicate the growth temperatures of cultures prior to extraction.

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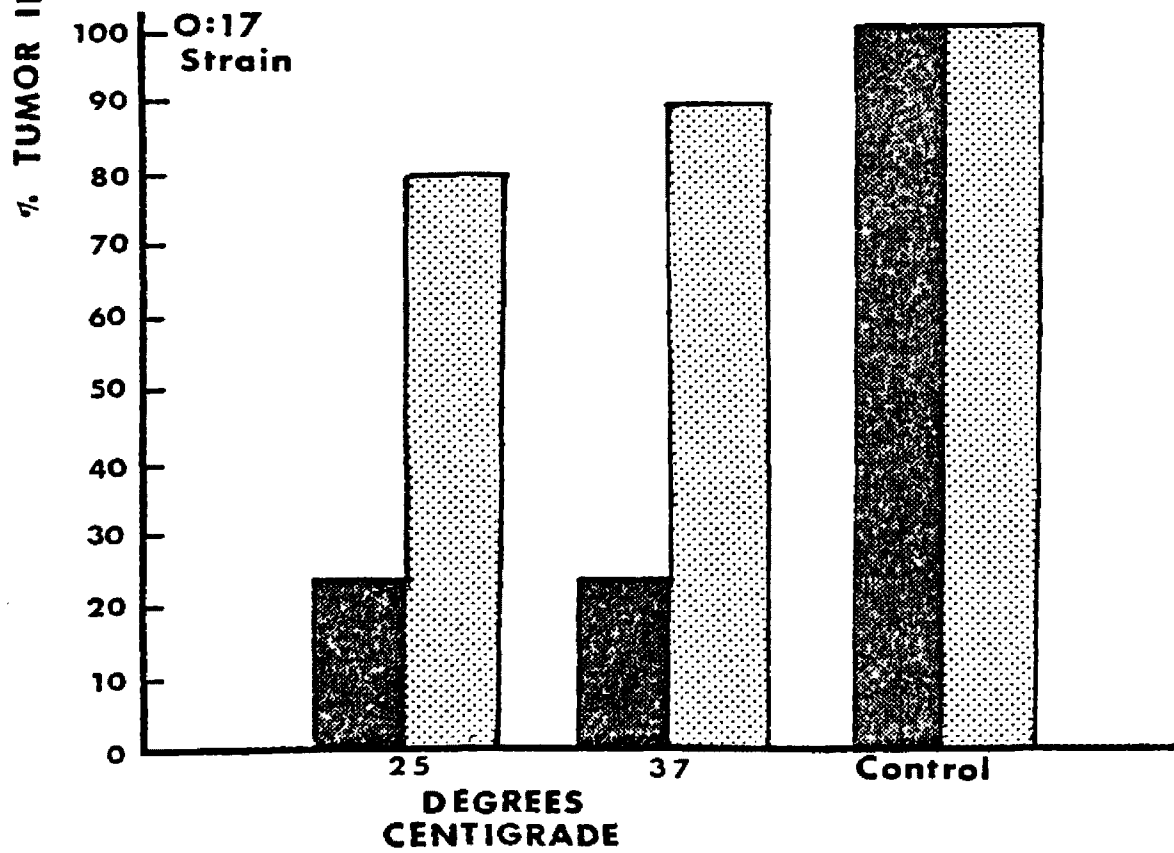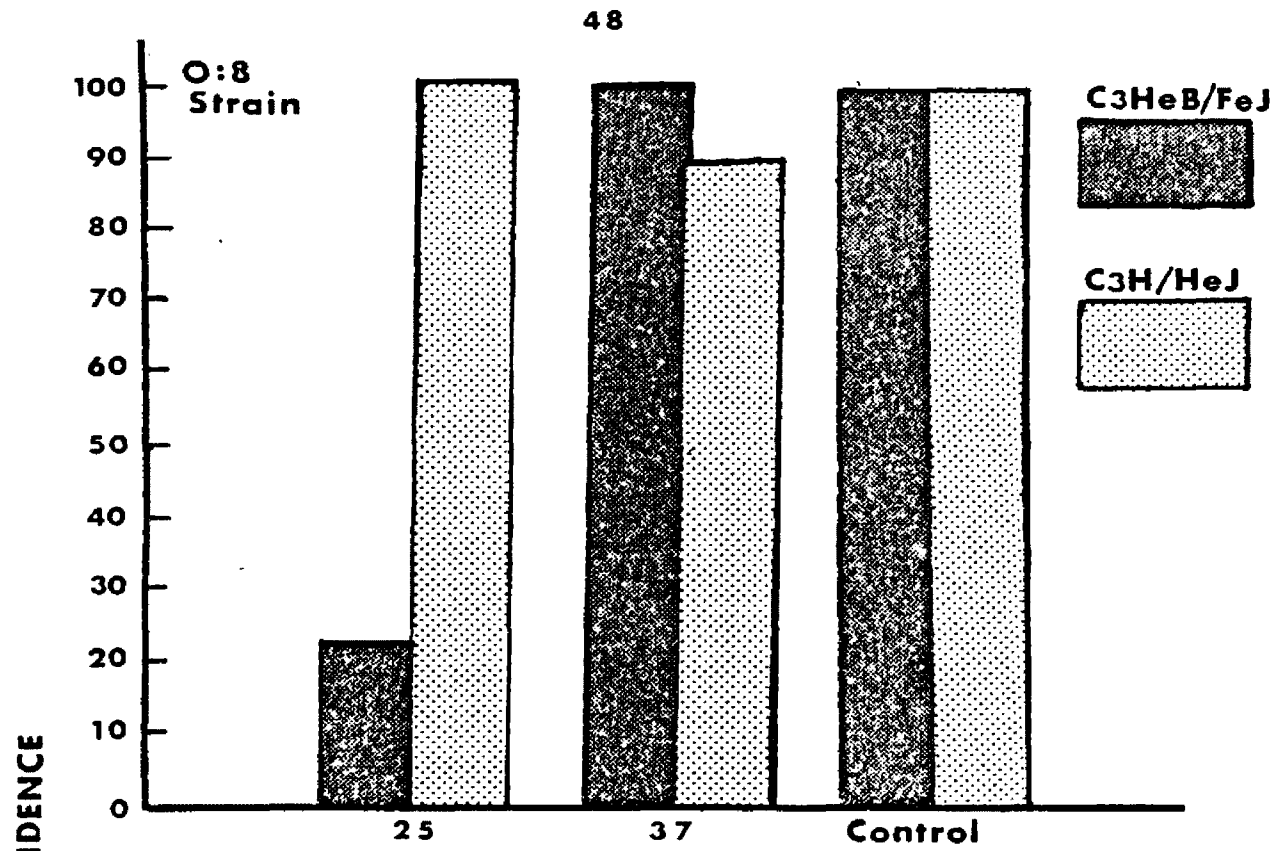
or LPS, from the O:8 and O:17 strains are presented in Table 8. For comparison, the activities in these same assays of Ec 434 are given. It can be seen that LPS from Y. enterocolitica pathophysiologically resembled LPS from E. coli. Since Table 4 demonstrated that the chemical composition of these materials were comparable to that of LPS from E. coli, the collective data indicated that a sedimentable, water extractable, biologically potent LPS was obtained from each of the Y. enterocolitica strains, grown at each temperature, by aqueous-phenol extraction.

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TABLE 8. Summary of biological activities of LPS from E. coli 0113 (Ec 434) and from Y. enterocolitica O:8 and O:17 strains grown at 25 C.

Biological assay	Ec 434	O:8 LPS	O:17 LPS
MLD_{50}[a]	$0.01\ ug$[b]	0.08 ug	0.054 ug
$CELD_{50}$	$0.012\ ug$[b]	0.002 ug	0.002 ug
FI_{40}	$0.19\ ug$[b]	0.18 ug	<0.02 ug
Mitogenicity[c]			
C3HeB/FeJ[d]	6.28	5.21	5.95
C3H/HeJ[d]	1.73	1.69	3.39
nude[e]	5.46	3.61	3.89

[a]For Ec 434 determined in C3HeB/FeJ mice potentiated with 12.5 ug/ml Actinomycin D; for LPS extracts from Y. enterocolitica determined in white mice (Rocky Mountain Laboratory, Hamilton, MT) potentiated with 10 ug/ml Actinomycin D.

[b]After Von Eschen et al. (102).

[c]Dose at 10 ug/ml; data reported as an average stimulation index.

[d]Jackson Laboratories, Bar Harbor, ME.

[e]Rocky Mountain Laboratory, Hamilton, MT.

CHAPTER IV

DISCUSSION

LPS from gram-negative bacteria was shown to be a heterogenous group of molecules whose physical, chemical, and biological characteristics varied somewhat from ones species to another, and, within a species, from one extraction method to another (4,55). Generally, however, all LPS molecules, regardless of species of origin, shared some common properties. They were usually more or less water soluble materials, which always contained phosphorous, carbohydrates, and fatty acids, and exhibited characteristic pathophysiological changes when injected into animals (4). Since no one property was established as inclusive for all LPS molecules, and exclusive of all other bacterial substances, the probability of identifying a substance as LPS increased directly with the number of chemical entities and pathophysiological responses demonstrated. Identification of significant amounts of KDO and hexosamine, relatively large amounts of carbohydrates, and a variety of fatty acids were distinctive chemical features of LPS from the Enterobacteriaceae (4). Toxicity and pyrogenicity for animals, the latter especially in primates and rabbits, production of Sanarelli-Shwartzman reactions, mitogenicity for B-lymphocytes, hemorrhagic necrosis of transplantable tumors, and adjuvanticity for protein antigens were some of the biological activities of LPS (4).

Table 3 shows that the fraction 3 extracts from the Y. enteroco-

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<u>litica</u> strains fit the chemical description of LPS. Significant concentrations of KDO and hexosamine were found. Also, these extracts were comprised almost exclusively of carbohydrates and lipids, with some phosphorous present. Examination of Tables 5 through 8 and Figures 3 through 6 revealed that the biological activities of fraction 3 materials were likewise consistent with the description of LPS. These materials were toxic for mice and 11 day old chick embryos, pyrogenic for rabbits, and mitogenic for B-lymphocytes and suppressive of tumor development in LPS responsive mice, but not in LPS nonresponsive mice. Furthermore, the fraction 3 materials were, by definition, the sedimentable, water soluble fraction recovered in phenol-water extraction. Thus, it was concluded that this fraction represented extractable LPS recovered from <u>Y. enterocolitica</u>.

The information derived from this study also was pertinent to two major topics of discussion regarding <u>Y. enterocolitica</u>. First, the taxonomical status of <u>Y. enterocolitica</u> is a subject of controversy. This problem concerns both the relationship of <u>Y. enterocolitica</u> strains to each other, and to the genus <u>Yersinia</u> and its relationship to the Enterobacteriaceae. In the first case, subspeciation of <u>Y. enterocolitica</u> was proposed on the basis of interrelationships of certain properties possessed by various clusters of strains. For instance, Chester and Stotzky (28) and Chester <u>et al</u>. (29) demonstrated the biochemical and immunological uniqueness of some rhamnose-positive strains of <u>Y. enterocolitica</u>. They suggested creation of a separate species for these strains, but also acknowledged that true rhamnose fermenting strains of <u>Y. enterocolitica</u> existed. Brenner <u>et al</u>. (19) suggested

subdivision of the species into at least 3 different species on the

basis of DNA hybridization homologies and biochemical reactivity. Two

of these species contained the atypical, rhamnose-positive strains.

The Y. enterocolitica strains used in this study were chosen

for, among other reasons, their different abilities to ferment rham-

nose. The remarkable similarities in chemical composition and biolo-

gical activities of LPS from these strains indicated that they were

related. Even the variations in the % yield data and % KDO content,

observed at the different growth temperatures, were, generally, in the

same direction (see Tables 1 and 2). Cells grown at 25 C yielded more

LPS, on a percent basis, than did cells grown at 37 C. Also, more

KDO was obtained, on a percent basis, from LPS from 25 C cultures

than was obtained from 37 C cultures. Table 3 shows that, generally,

the chemical composition of LPS preparations from the two strains re-

sembled one another. In pathophysiological responses there was a

similar correspondence. Figure 3 demonstrated that the dose-fever

responses in rabbits of LPS from both strains were analogous. The dose-

mitogenic responses of LPS from the two strains, given in Figures

4 and 5, also displayed a good correspondence.

However, several minor physical and chemical differences between

the strains were detected. Table 1 shows that the O:17 strain yielded

less water extractable materials than did the O:8 strain. Approxi-

mately 4 times as much LPS was extracted from the O:8 strain as from

the O:17 strain at either growth temperature. Table 3 demonstrates

that the carbohydrate content of LPS from the two strains differed.

LPS from the O:17 strain contained a significantly larger carbohy-

drate content than did the O:8 strain, regardless of the growth temperature. Furthermore, it was noted that the LPS from the O:17 strain contained a significant concentration of DDH(s). No DDHs were detected in the O:8 strain, however. Nevertheless, these observed differences in extractability pattern, and carbohydrate and DDH content probably represented acceptable physical and chemical strain variations in the LPS. The sum of these observations overwhelmingly supports the relationship of the strains to one another, despite their chemical differences.

The relationship of the genus Yersinia, and especially Y. enterocolitica, to the Enterobacteriaceae also is a subject of controversy. Previous studies implied a closer relationship of Y. enterocolitica than of Y. pestis or Y. pseudotuberculosis to the Enterobacteriaceae (20,71). This led to speculation as to the correctness of inclusion of Y. enterocolitica with the latter two species (19,20,68,71). DNA hybridization studies showed a relatedness of this bacterium to the Enterobacteriaceae, but conflicting evidence on relatedness of the other two Yersinia species to the Enterobacteriaceae (19,71). Maeland and Digranes (64) showed that Y. enterocolitica, and one strain of Y. pseudotuberculosis, possessed the common enterobacterial antigen, an antigen characteristic of the Enterobacteriaceae. Antigenic relationships of Y. enterocolitica to Vibrio cholerae (12,85), Proteus morganii (101), Brucella species (12,48,66,85), and Salmonella species (10,30) were found. However, antigenic cross-reactivity of Y. enterocolitica to Y. pestis also was demonstrated (6,10). Barber and Eylan (10,11) showed that antigenically related strains of Y.

enterocolitica and Y. pestis did not demonstrate the same antigenic relationship to Salmonella and Shigella. They found that Y. enterocolitica antigens cross-reacted relatively strongly with Salmonella and Shigella antisera, but Y. pestis antigens cross-reacted weakly or not at all. Acker (1) and Acker and Wartenberg (2) demonstrated that LPS from Y. enterocolitica was structurally similar to that of E. coli and S. typhimurium in electron micrographs. Kanamori (55) showed that LPS from Y. enterocolitica was similar to identically extracted LPS from E. coli in biological activities and chemical composition.

The results of this study agreed with those reported by Kanamori (55). Water soluble materials from both strains were obtained whose chemical compositions and biological activities parallelled those of LPS from E. coli, extracted and characterized by similar methods. Thus, on the basis of LPS similarity, the relationship of these Y. enterocolitica strains to the Enterobacteriaceae, represented by E. coli, was established. Furthermore, recovery of DDH(s) from the LPS of the O:17 strain implied a possible relationship of this strain to Y. pseudotuberculosis. Recovery in the latter species of DDH(s) was reported (84,95). However, DDHs were not demonstrated previously in LPS from Y. enterocolitica, although unusual deoxyhexoses were found (13,36,103).

The second major subject of controversy surrounding Y. enterocolitica concerns the association of various factors with strain virulence. Several of these factors were strain dependent, such as serotype, biochemotype, and source of isolate. With regard to serotype, certain

Y. enterocolitica serotypes were found to be invasive in tissue culture or enterotoxigenic, regardless of their source (76,83,100). Biochemically, the ability of some strains to ferment rhamnose was associated with low, or no, experimental pathogenicity. This was true in studies dealing with tissue culture invasiveness and enterotoxin production of Y. enterocolitica strains (63,76). However, rhamnose fermenting strains were recovered from clinical specimens, and in some cases were the apparent agent of disease (16,93). Source of an isolate also was reported to be related to production of experimental pathogenicity. Human isolates were reported to be pathogenic for mice, invasive in tissue culture, and/or enterotoxin producers (7,14,27,63,65,76). However, nonhuman isolates also were reported to be active in some of these, or other, assay systems (76,77,97,98).

The strains used in this study were expressly chosen for their dissimilarities with regard to the above characteristics. One was a serotype O:8, rhamnose-negative, human spleen isolate, and the other was a serotype O:17, rhamnose-positive, water isolate. Examination of the toxic, pyrogenic, mitogenic, and tumor suppressive activities of LPS from these strains revealed no significant strain dependent variation. However, a strain dependent variation in LPS production was observed. Examination of Table 1 shows that 4 times as much LPS was obtained from the O:8 strain as from the O:17 strain, regardless of the growth temperature. Thus, the pathogenicity of different Y. enterocolitica strains may be related to the amount of endotoxin they produce. Also, a strain dependent variation in the biological activities of the fraction 1 and 4 materials occurred. Generally, the

0:17 strain preparations were more toxic for mice and chick embryos and more pyrogenic for rabbits, regardless of growth temperature. For example, the fraction 4 material of the 0:8 strain grown at 25 C exhibited a MLD_{50} of 0.4 ug, a $CELD_{50}$ of 0.035 ug, and a FI_{40} of 0.58 ug. The same fraction at the same growth temperature from the 0:17 strain displayed a MLD_{50} of 0.021 ug, a $CELD_{50}$ of 0.0024 ug, and a FI_{40} less than 0.04 ug. This was a 10-fold difference, or better, in potency for each assay from the 0:8 strain. However, this relationship did not extend to the mitogenic activities of these fractions. Fraction 1 and 4 materials elicited similar dose-mitogenic response curves in the test mouse strains, regardless of the Y. enterocolitica strain (data not shown). Nevertheless, the greater biological responsiveness of the fraction 1 and 4 extracts from the 0:17 strain suggests the possible existence of an additional toxic factor(s) in this strain.

Another factor associated with pathogenicity of strains was growth temperature of the bacterium. Temperature-dependent variations in experimental pathogenicity for mice (26) and lice (60), invasiveness for HeLa cells in vitro (63), and production of enterotoxin (75) were observed. Cells grown at 25 C were more active in these assays than ones grown at 37 C. However, other reports found no growth temperature-dependent effects upon pathogenicity (77,80).

In this study, with one exception, no temperature-dependent difference in absolute biological activities of the LPS from either strain was demonstrated. The exception was the tumor suppression activity of crude LPS from the 0:8 strain grown at 37 C (see Figure 6). The

concomitant increase in the % protein (see Table 3) observed in the purified LPS from this strain could be related to this loss of tumor suppression activity at the higher temperature. However, it might also be due to some other factor(s), or lack of it, in the extract.

Although no significant variations in the absolute potencies of the LPS extracts from these strains were discernible, the amounts of LPS extracted from cells grown at the two temperatures were different. For each strain, up to 4 times as much LPS was extracted from cells grown at 25 C as opposed to ones grown at 37 C. So, when the number of MLD_{50}s, $CELD_{50}$s, and FI_{40}s per 1.0 mg of cells cultivated at each temperature were calculated using the % yields of the fraction 3 extracts, a marked difference in biological potency was found. The data from these calculations are given in Table 9. It can be seen that LPS from each strain grown at 25 C contained more potency per cell weight than did cells grown at 37 C. For instance, for the O:8 strain there were 299 FI_{40} doses per 1.0 mg of cells grown at 25 C, but only 9 FI_{40} doses per 1.0 mg of cells grown at 37 C. For the O:17 strain the ratio was 685:1 FI_{40} doses per 1.0 mg of cells grown at 25 C as opposed to 37 C. The number of MLD_{50}s and $CELD_{50}$s also displayed a similar relationship. Carter (25) observed that endotoxin from the WA strain grown at 37 C was less potent on a per weight basis than that from S. typhi. The results obtained in this study were consistent with his observation. Thus, the experimental evidence showed a temperature-dependent variation in potency of LPS preparations from Y. enterocolitica directly related to production of LPS and not to its absolute potency.

TABLE 9. The number of $MLD_{50}s$, $CELD_{50}s$, and $FI_{40}s$ of LPS per 1.0 mg of Y. enterocolitica cells grown at 25 or 37 C.

Bacterial strain	Growth temperature[a]	# $MLD_{50}s$	# $CELD_{50}s$	# $FI_{40}s$
0:8	25	674	28,824	299
	37	306	2,227	9
0:17	25	254	6,850	685
	37	17	943	1

[a] In degrees centigrade.

Therefore, the potential for exertion of a direct toxic effect on the host by LPS in Y. enterocolitica infection seems plausible. Kanamori (55) also suggested as much. The pathophysiological response described in mice injected ip with the bacterium was consistent with a diagnosis of endotoxicosis (25). Even without a direct expression of endotoxicity in infection, the possibility of post-infectious auto-antibody response in the host through adjuvant action of the LPS, as described by Gripenberg et al. (38), exists. Thus, endotoxin from Y. enterocolitica could contribute to its clinical pathogenicity.

Several conclusions were drawn from this study of LPS from Y. enterocolitica. Characterization of LPS was used as a taxonomic tool in other studies (1,2,4). The similarities of the LPSs from the two strains to one another and to LPS from E. coli indicated a relation-ship of the strains to one another and to the Enterobacteriaceae. The identification of DDH(s) in the LPS from the O:17 strain suggested a relationship of this strain to Y. pseudotuberculosis. Comparison of the toxicity, pyrogenicity, and immunogenicity of the LPS extracts from Y. enterocolitica to those of LPS from E. coli indicated that LPS from Y. enterocolitica was as biologically active as LPS from the Enterobacteriaceae. This suggested that endotoxin could play a role in infection due to Y. enterocolitica. The expression of a growth temperature-dependent variation in LPS production was consistent with known temperature-dependent effects upon pathogenicity of this organism. Therefore, a facet of increased infectivity for humans, or other hosts, of strains grown at 25 C could be increased endotoxin production.

SUMMARY

Isolation of a sedimentable, water extractable LPS from each of the Y. enterocolitica strains at both growth temperatures was accomplished. These LPS extracts were similar in chemical composition and biological activities to a similarly extracted LPS from E. coli. Furthermore, they were similar to one another in these characteristics and in extractability pattern.

The data collected in this study indicated a taxonomical relationship of the strains to one another, to the Enterobacteriaceae, as represented by E. coli, and, in a limited way, to the genus Yersinia. Generally, strain dependent variation in biological activities of the LPS was not observed. However, a temperature-dependent effect upon LPS production was observed, although there were no differences in the absolute potencies of the LPS of either strain at either growth temperature. As measured by several assay systems, the relative potency of the LPS was greater for bacteria grown at 25 C than at 37 C. Since some Y. enterocolitica strains were observed to be more pathogenic experimentally in several assay systems when grown at 25 C than at 37 C, the increased production of LPS at 25 C may be significant in clinical infection due to this organism.

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