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Composition of the Antigenic Material Removed from *Campylobacter jejuni* by Heat

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The antigenic material removed from *Campylobacter jejuni* by the boiling of whole cells in saline was examined biochemically. Analyses showed that the extracted material contained 3 μg of protein per ml per mg of wet cells and ca. 2.6 μg of carbohydrate per ml per mg of wet cells. Further extraction of the material with chloroform-methanol produced about 0.5 μg of water-insoluble residue per ml per mg of wet cells, suggesting the presence of lipid as well. Additional analyses revealed the presence of hexose, pentose, heptose, hexosamine, and 2-keto-3-deoxyoctonic acid, and the extract was also positive by the *Limulus* amoebocyte lysate assay for lipopolysaccharide. An examination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that at least 10 different protein bands could be detected. One of the major bands corresponded to the major outer membrane protein, as determined by comparison with an outer membrane protein preparation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Another major protein in the heated extract corresponded to a band previously shown to be flagellin. An analysis of the time course of the release of material showed that a significant amount was removed after 3 to 10 min at 100°C, but the release of material seemed to be delayed at lower temperatures. These results show that the treatment of *C. jejuni* with heat produces a complex mixture of components, including cell wall lipopolysaccharide, the major outer membrane protein, and flagellin. It is likely that some cytoplasmic components are present as well. Blebs of outer membrane have been observed with this organism by electron microscopy. Our results confirm this and suggest that the heating of cells accelerates this blebbing process.

A number of different serological typing schemes have been developed (1, 15, 20, 22) to assist in epidemiological investigations of *Campylobacter jejuni*. One of the problems in developing and comparing these schemes is that the antigens of this organism have not been fully characterized. In early studies with *C. fetus*, Wiidik and Hlidar (26), Ristic et al. (21), and Berg et al. (2) described two basic antigens, one removed by heat (i.e., heat labile) and assumed to be a capsule and another that remained with the cells after they were heated (i.e., heat stable) and that was assumed to be the lipopolysaccharide O antigen. This arrangement has not been confirmed with *C. jejuni*, however. In a recent investigation, Abbott et al. (1) concluded that the material removed by the heating of *C. jejuni* represented flagella. On the other hand, Penner and Hennessy (20) concluded that this material was the lipopolysaccharide cell wall O antigen, because material with similar antigenicity could be removed by treatment with EDTA, a method used to remove endotoxin (14, 18).

As this antigenic material is the basis for the Penner typing scheme (20) and as this scheme is becoming widely used, the objective of the experiments reported here was to examine the composition of this material in greater detail.

MATERIALS AND METHODS

Bacteria. The *C. jejuni* strains used in this study were clinical isolates obtained from the Clinical Microbiology Laboratory of The University of Texas Medical Branch, Galveston. They were identified by established procedures (10) and biotyped by the procedures described by Hébert et al. (9). All of the strains used were biotype 3, except strain 1200, which was biotype 1. The organisms were stored at −70°C in skim milk.

Antigen preparation. Frozen stock cultures were thawed, inoculated onto 5% sheep blood agar plates, and incubated at 37°C for 48 h under microaerophilic conditions achieved either by evacuation-replacement or with CampyPak envelopes (BBL Microbiology Systems, Cockeysville, Md.). The bacteria were then subcultured to freshly prepared Columbia blood agar base without blood and incubated under the same conditions for 24 h. At this time, the cells were removed from the surfaces of the plates, suspended in 0.9% pyrogen-free saline at ca. 100 to 150 mg/ml (wet weight) and, in most experiments, boiled for 60 min; however, in some experiments shorter times and lower temperatures were used. The cells were removed from the boiled preparation by being centrifuged at 10,000 × *g* and filtered through a 0.2-μm filter (Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.).

Biochemical analyses. The protein content was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) (4). Total carbohydrate was measured by the phenol-sulfuric acid method (17). The lipid content was estimated by reextracting the antigen preparation with methanol-chloroform, as described by Kates (12), and then weighing the water-insoluble residue obtained. Hexose was measured by the H_2SO_4-cysteine method described by Dische et al. (7), and the modified phloroglucinol reaction described by Dische (5) was used to measure aldopentoses. The methylpentose content was determined as described by Dische and Shettles (6). Heptose was measured by the more sensitive modification of the cysteine-sulfuric acid method described by Wright and Rebers (27). 2-Keto-3-deoxyoctonic acid (KDO) was measured by the thiobarbituric acid assay developed by Karkhanis et al. (11), the hexosamine content was measured as described by Strominger et al. (23), and the phosphate content was analyzed as described by Dryer et al. (8).

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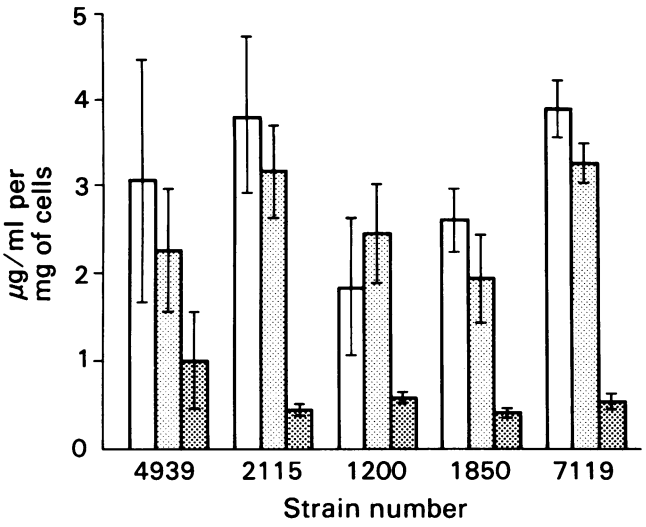

FIG. 1. Analyses of protein (□), carbohydrate (▨), and lipid (⊡) in five different *C. jejuni* strains. The results represent the average of three determinations expressed as micrograms per milliliter per milligram of wet cell weight. The vertical lines indicate standard deviations.

***Limulus* amoebocyte lysate assay.** A series of 10-fold dilutions, from 10^{-4} to 10^{-9}, were prepared from the heat-treated, cell-free extract with pyrogen-free water. *Limulus* amoebocyte lysate (Difco Laboratories, Detroit, Mich.) was added to each dilution by a standard technique. Negative controls, consisting of saline and tubes treated similarly as those used for extraction, were included in each experiment.

Isolation of outer membrane proteins. The method used for the isolation of outer membrane proteins was that described by Logan and Trust (16), except that the bacteria were fractured with a Ribi Press (Ivan Sorvall, Inc., Norwalk, Conn.).

Polyacrylamide gel electrophoresis. Electrophoresis was performed essentially as described by Laemmli (13), with 5% stacking gels and 10% separation gels. Antigen protein (ca. 25 μg) was placed in each well of each gel, and a 20 mA current was applied for ca. 4 h. The molecular weights of the proteins were estimated with the following molecular weight standards: phosphorylase *b* (94,000 [94K]), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (20K), and α-lactalbumin (14K). These were supplied as a kit (Pharmacia Fine Chemicals, Piscataway, N.J.). The gels were stained with Coomassie brilliant blue as described by Weber and Osborn (25).

RESULTS

Biochemical analyses. The results of protein, carbohydrate, and lipid analyses of extracts from five *C. jejuni* strains are shown in Fig. 1.

Table 1 shows the results of analyses for specific carbohydrate components and phosphate. Significant amounts of hexose and pentose were detected; smaller amounts of heptose, hexosamine, and KDO were found, and there was a small amount of detectable phosphate. Essentially no methylpentose could be detected, except for a very small amount in strain 1200.

Protein components of the antigen preparation. The number of protein components was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The number of components depended upon the particular isolate, but most isolates produced at least 10 different bands (Fig. 2).

TABLE 1. Carbohydrate components of the antigen removed by heat from *C. jejuni*

Component	Mean concn (±SD)[a]
Total carbohydrate	2.2 ± 0.34
Hexose	0.87 ± 0.26
Methylpentose	0.04 ± 0.06
Pentose	1.02 ± 0.57
KDO	0.15 ± 0.03
Heptose	0.12 ± 0.05
Hexosamine	0.24 ± 0.08
Phosphate	0.05 ± 0.01

[a] Mean of duplicate determinations for six *C. jejuni* strains expressed as micrograms per milliliter per milligram of wet cell weight.

Reactivity with *Limulus* amoebocyte lysate. Antigen extracts from five strains were tested for the ability to cause gelation of *Limulus* amoebocyte lysate. Dilutions of the extracts from 10^{-4} to 10^{-9} in pyrogen-free water were tested. For all extracts a positive reaction occurred at dilutions of up to 10^{-6}.

Comparison with purified outer membrane protein preparation. An outer membrane preparation from strain 2115 contained two major bands (Fig. 3, lane 1) with molecular weights of 43K and 58K. A control preparation (Fig. 3, lane 2) consisting of the filtrates from cells that had been agitated briefly in saline but not heated contained a faint band corresponding to the 43K protein, in addition to several other fainter bands. The filtrates from boiled cells produced bands corresponding to the 43K and 58K bands, although after being heated for 60 min, the higher-molecular-weight component seemed to have an actual molecular weight slightly higher than 58K.

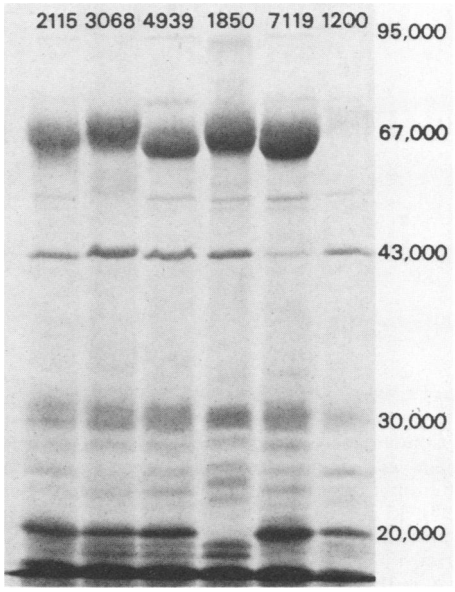

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of heat-extracted antigen from six *C. jejuni* strains. The gel was stained for protein with Coomassie brilliant blue. The strain numbers are shown at the top, and the approximate molecular weights of the major bands are shown at the right. At least 10 different proteins were detected with most strains. All isolates had a major 43K band, and most also had major 67K and 20K components.

Time course of release. The protein, carbohydrate, and KDO released after treatment for different times up to 60 min were examined (Fig. 4). Significant variation occurred between replicate experiments for protein and total carbohydrates, but the results indicate a considerable removal of these materials by 3 to 10 min.

Effect of temperature on extraction. To examine the temperature dependence of extraction, we conducted extraction experiments at 60 or 80°C. An analysis of the material removed at 60°C by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 5) showed a number of bands, with a progressive increase in the density of the individual bands. A band corresponding to the 43K outer membrane protein was present, but there was no heavy band corresponding to the 58K protein. At 80°C the density of the bands was more uniform, and the 43K band was found in all preparations. The 58K band was present, but not until after 10 min of treatment. In preparations heated for 10 min or longer the number of bands decreased. A quantitative analysis of material removed at 60°C at different times (Fig. 6B) showed less variability than at 100°C, and less total KDO was removed. There appeared to be some slight delay in the release of material, but essentially all was removed within ca. 10 min of treatment at 60°C. The results of treatment at 80°C (Fig. 6A) were similar to the results obtained at 100°C.

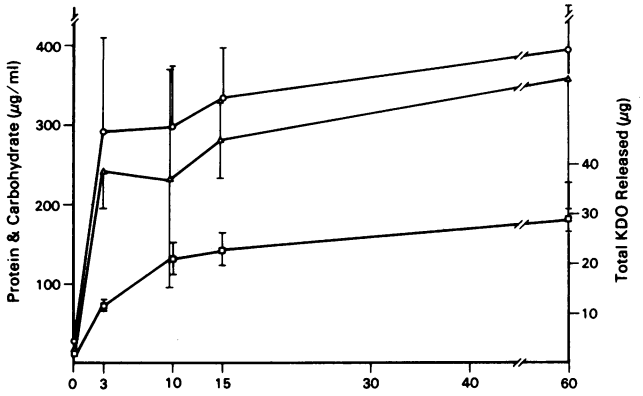

FIG. 4. Removal of total protein (○), total carbohydrate (△), and KDO (□) after treatment of *C. jejuni* 2115 at 100°C for various amounts of time. Each point is the mean of three determinations, and the vertical bars show standard deviations. The time in minutes is shown along the horizontal axis. There was substantial variability in the removal of protein and total carbohydrates, but a significant amount of material was released after even 3 min of treatment. KDO was only partially released after 3 min, but essentially all that could be removed was extracted after 10 to 15 min. Determinations for zero time were made from filtrates from cells agitated in saline with a vortex mixer (Scientific Industries, Inc., Bohemia, N.Y.).

DISCUSSION

The results of our investigation show that the material removed from *C. jejuni* by boiling of the cells is a complex mixture of components. Analyses showed that the material contains at least 10 different proteins and a number of carbohydrate components as well, including hexose, pentose, heptose, hexosamine, and KDO. A small amount of phosphate was also detected. Further extraction of the boiled preparation with methanol-chloroform yielded water-insoluble material suggestive of lipids, but more work is necessary to confirm this and identify the specific lipid components. The antigen preparation also showed a high degree of reactivity with *Limulus* amoebocyte lysate. A comparison of the extract with a purified outer membrane protein preparation revealed two major bands in common. The 43K band has been identified by other investigators (3, 16) as the major outer membrane protein. The other major band in common, the 58K protein, is virtually identical to the

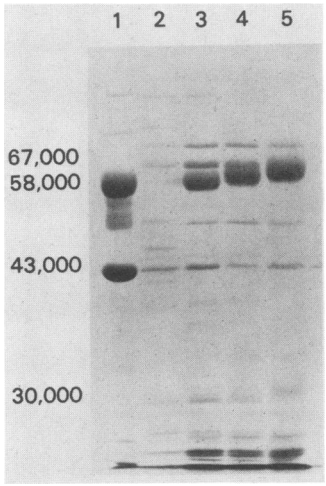

FIG. 3. Comparison of an outer membrane protein preparation from *C. jejuni* 2115 (lane 1) with extracts from the same strain removed after brief agitation in saline (lane 2) or heating at 100°C for 3 min (lane 3), 15 min (lane 4), or 60 min (lane 5). The major outer membrane proteins had molecular weights of 58K and 43K. The 58K band occurred in heated preparations at a density approximately equal to that in the outer membrane protein preparation, but this was not the case for the saline extract. The 43K band occurred in all extracts but at a lower density than in the outer membrane protein preparation. Previous work (3, 16) has identified the 43K protein as the major outer membrane protein. The 58K protein is probably flagellin, as it was almost identical to the band reported to be flagellin by Logan and Trust (16). Molecular weights are shown at the left.

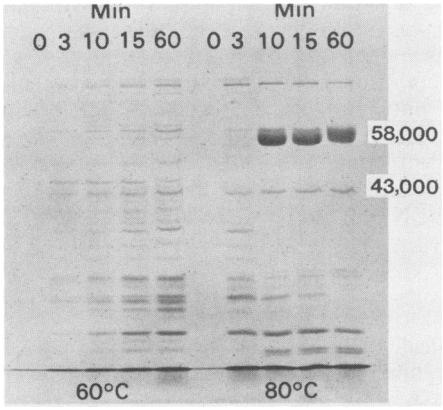

FIG. 5. Comparison by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein extracted after different amounts of time at either 60 or 80°C. At 60°C very little protein was removed after agitation in saline (zero time), but progressively more was removed with increasing time. The major outer membrane protein (43K) was clearly discernible and increased progressively. The 58K band was not removed, however. At 80°C the 43K band was found in all preparations at about the same density, but the 58K band was only removed after 10 min of treatment. A very similar band has been identified by others (16) as flagellin. Molecular weights are shown at the right.

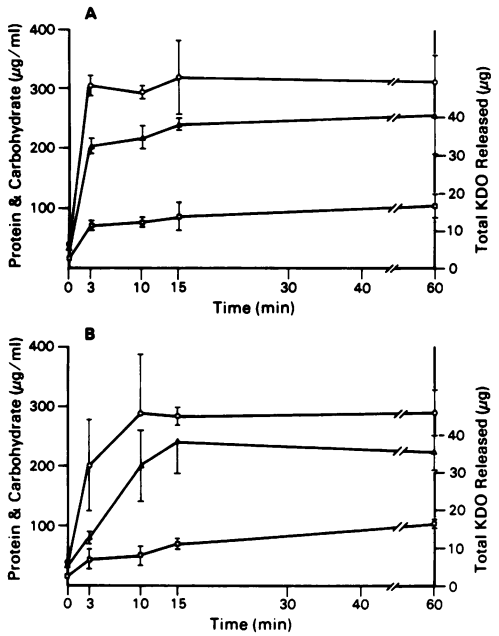

FIG. 6. Time course of removal of total protein (○), total carbohydrate (△), and KDO (□) after heating at 80°C (A) or 60°C (B). At 80°C essentially all the material that could be removed was extracted after 3 min of treatment. At 60°C the extraction was delayed slightly but appeared to be essentially complete by 10 to 15 min. The determinations made at zero time were from filtrates from cells agitated briefly in saline with a vortex mixer (Scientific Industries).

band described as flagellin in similar gels by Logan and Trust (16) and almost undoubtedly is flagellin. The actual molecular weight of 58K was slightly lower than the value they reported, but this could well be because of a slight variation in the electrophoresis procedure.

These data reaffirm the conclusion of Penner and Hennessy (20) that the heating of *C. jejuni* removes O antigen but also indicate that other components of the outer membrane are also removed. Observations by electron microscopy (19, 24) have shown that *C. jejuni* normally produces vesicles interpreted as blebs of outer membrane, and Blaser et al. (3) published evidence that outer membrane proteins are present in such preparations. As the removal of KDO and the major outer membrane protein appear to be temperature dependent, it appears that the process of release of the outer membrane protein is accelerated by heat. The removal seems to be limited, though, because measurements (unpublished observations) indicate that only about 10% of the amount of KDO in the cell is released by heat.

Our data also confirm observations by Abbott et al. (1) that flagella are removed by the heating of cells. They appear to be resistant to 60°C, but almost complete removal occurred after treatment for 10 min at 80°C. Extended heat treatment apparently alters the flagellin, either by extensive denaturation or aggregation with other proteins, so that the molecular weight increases, as indicated by gel electrophoresis. Prolonged heating also causes aggregation of other proteins, as shown by the fact that there were fewer bands in gels from preparations heated extensively.

Our results do not substantiate observations by Wiidik and Hlidar (26) and Ristic et al. (21) that the material removed by heat is a capsule, but because they examined *C. fetus*, the results of our investigation may not be strictly comparable.

In addition to flagella and outer membrane components, the heat-extracted material probably contains minor cytoplasmic components as well. Certainly, a number of proteins which have not been accounted for were found by gel electrophoresis, and drastic treatment, such as boiling, seems likely to break open the cells.

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