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Pasteurella multocida Produces Heat Shock Proteins in Turkeys

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***Pasteurella multocida* produces an acute septicemic disease of turkeys. Since turkeys have a normal body temperature of 42°C, it follows that *P. multocida* would produce heat shock proteins during the course of the infectious process. We show here that *P. multocida* produces several proteins at 42°C, but not at 32°C, and vice versa. Four of these proteins (70, 60, 40, and 35 kDa in molecular mass) were recognized by serum obtained from a turkey infected with *P. multocida*, suggesting that they were produced in vivo.**

Heat shock proteins are produced by eukaryotic and prokaryotic cells following exposure to elevated temperature. Some of these proteins serve as "chaperons" to other cellular proteins to assist in the folding, transport, and prevention of degeneration of these other proteins in cells stressed by increases in temperature or in other environments that may result in stress, e.g., within a macrophage (1, 9). Heat shock proteins are essential, therefore, for survival when organisms are subjected to increased temperatures.

Avian species, such as turkeys, normally have high body temperatures (40 to 42°C). We hypothesize that microorganisms, such as *Pasteurella multocida*, responsible for septicemic disease in turkeys (6) respond to tissue invasion with the production of heat shock proteins. We propose that heat shock proteins produced by bacteria affecting avian hosts may be virulence determinants, since without the ability to produce this unique class of protein, bacteria would be incapable of producing disease.

The present study was undertaken to determine whether *P. multocida* exhibits a heat shock response and to determine whether proteins produced as a part of this response are made in vivo.

P. multocida P-1059 (serotype A:3) was used. This strain was isolated from the tissues of a turkey that had died of avian cholera. Strain P-1059 was grown in short-term labelling medium (RPMI 1640 [GIBCO-BRL, Gaithersburg, Md.] plus 5% fetal calf serum and 2 mM glutamine) for 24 h at 32°C. Aliquots (1/2 ml) were then placed into each of five 0.9-ml microcentrifuge tubes. Non-heat-shocked samples were prepared in the following manner. [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) (100 μCi) was added to one tube, and after 15 min at 32°C, the bacteria were centrifuged (15,600 × *g*, 5 min, room temperature) and the labelling medium was removed. Heat-shocked samples were prepared in the following manner. The tubes were shifted to 42°C, and after 5, 10, 30, and 60 min, 100 μCi of [^{35}S]methionine was added to each tube. After a 15-min incubation, the bacteria were centrifuged and the labelling medium was removed. Bacteria were lysed in two-dimensional gel sample buffer consisting of 9.5 M urea, 2% Nonidet P-40, 2% ampholytes (Bio-Lyte 5/7 [Bio-Rad Laboratories, Richmond, Calif.] and 2-D Pharmalyte [Pharmacia LKB, Uppsala, Sweden]), and 5% 2-mercaptoethanol. An equal volume of glass beads (212- to 300-μm diameter; Sigma Chemical Co., St. Louis, Mo.) was then added to each tube. After the tubes were vortexed for 3 min, they were centrifuged (15,600 × *g*, 5 min, room temperature). The proteins in the supernatants were separated by two-dimensional gel electrophoresis. Two-dimensional polyacrylamide gel electrophoresis was performed as described elsewhere, with modifications (5). Briefly, proteins were separated in the first dimension according to their isoelectric points in tube gels containing 3% acrylamide and 1.5% ampholytes (pH 5 to 7 [Bio-Rad] and pH 3.5 to 10 [Pharmacia LKB]) mixed in a 4:1 ratio. Proteins were separated in the second dimension by electrophoresis through a 3% stacking gel and a 10% separating gel.

Proteins were transferred (Trans-Blot; Bio-Rad Laboratories) to nitrocellulose membranes (Schleicher and Schuell, Keene, N.H.) (8). Membranes were wetted in TBS (0.02 M Tris base, 0.5 M NaCl) and then incubated first for 1 h in blocking solution (TBS plus 3% gelatin) and then in primary antibody (1:250 dilution in TBS with 1% gelatin) overnight. The blots were then washed twice with TTBS (TBS with 0.05% Tween 20 [vol/vol]) and incubated in secondary antibody (1:1,000 dilution of peroxidase-labelled goat anti-turkey immunoglobulin G [Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.]). Blots were washed twice with TTBS and once with TBS prior to color development by the addition of H_2O_2 and 4-chloro-1-naphthol.

The primary antibody was either serum from a 6-week-old Nicholas Broad Breasted White turkey that had not been exposed to *P. multocida* (naive serum) or serum obtained from a 6-week-old Nicholas Broad Breasted White turkey that had been given live *P. multocida* T13 (serotype A:3,4) orally and then inoculated intravenously with the same strain (postexposure serum).

Autoradiography was performed by exposing the nitrocellulose blots to X-ray film (Kodak X-Omat; Sigma) at room temperature for 36 h.

P. multocida shifted to 42°C conditions produced several proteins in increased amounts compared with amounts produced at 32°C (Fig. 1 and 2). On the other hand, the production of some proteins decreased at the higher temperature. After 1 h at increased temperature, most of the cellular

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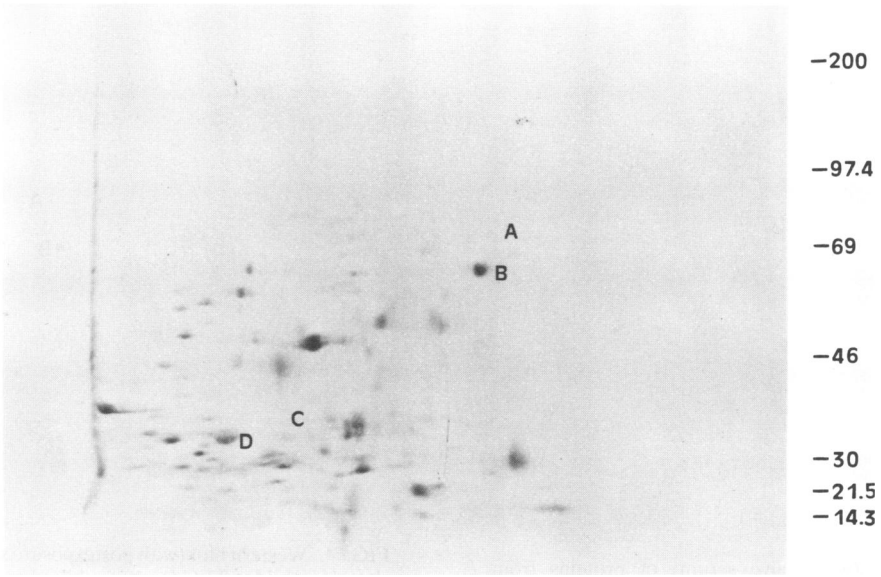

FIG. 1. Autoradiograph of [^{35}S]methionine-labelled proteins from *P. multocida* grown at 32°C for 18 h, labelled at 32°C for 15 min, and then separated by two-dimensional polyacrylamide gel electrophoresis. Capital letters A through D represent the locations of the immunoreactive proteins shown in Fig. 4. Numbers on the vertical axis represent the positions of migration of known proteins: myosin (200 kDa); phosphorylase *b* (97.4 kDa); bovine serum albumin (69 kDa); ovalbumin (46 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (21.5 kDa); lysozyme (14.3 kDa) (^{14}C-labelled Rainbow Protein molecular weight markers; Amersham Corp.).

proteins, including those whose production had increased, were no longer produced (data not shown).

In order to assess the immunological reactivity of those proteins whose production was influenced by an increase in temperature, Western blots (immunoblots) with serum from a turkey previously exposed to *P. multocida* were performed. No immunologically reactive proteins were detected with naive serum (Fig. 3). However, immunologically reactive proteins, including some whose production was influenced by the increase in temperature, were detected by postexposure serum. In particular, four proteins, labelled A, B, C, and D (Fig. 4), elicited an antibody response. Two of these proteins, represented by spots A (70 kDa) and C (40 kDa), were not produced at 32°C but were produced at 42°C. The other two immuno-

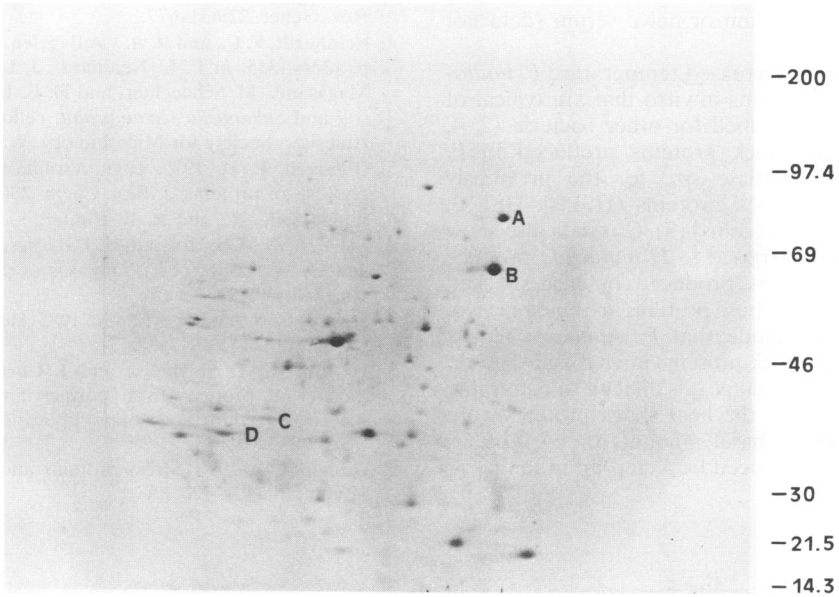

FIG. 2. Autoradiograph of [^{35}S]methionine-labelled proteins from *P. multocida* grown at 32°C for 18 h, shifted to 42°C for 10 min, labelled for 15 min at 42°C, and then separated by two-dimensional polyacrylamide gel electrophoresis. Capital letters A through D represent the locations of the immunoreactive proteins shown in Fig. 4. Numbers on the vertical axis represent the positions of migration of known proteins (see the legend to Fig. 1 for details).

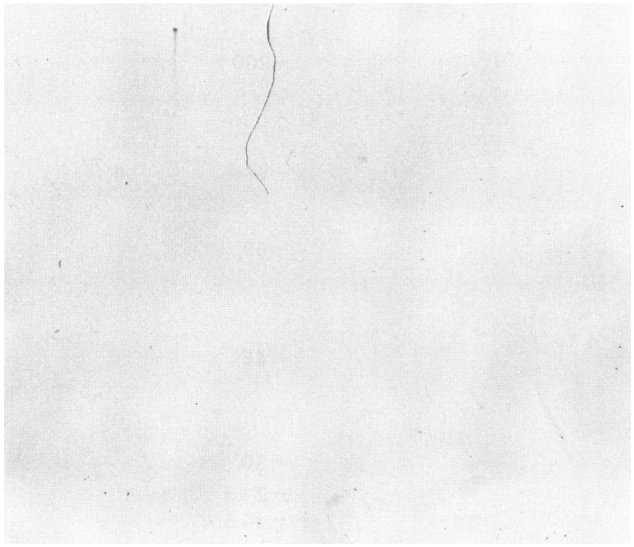

FIG. 3. Western blot (with naive serum) of proteins from *P. multocida* grown at 32°C for 18 h, shifted to 42°C conditions for 10 min, labelled for 15 min at 42°C, and then separated by two-dimensional polyacrylamide gel electrophoresis. No immunologically reactive proteins were detected.

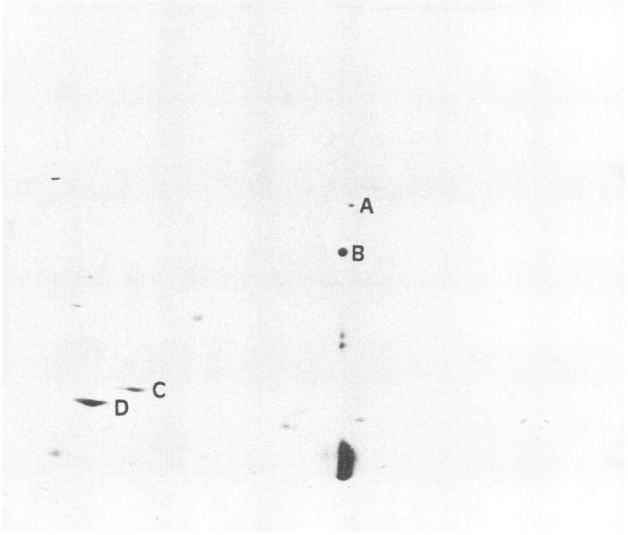

FIG. 4. Western blot (with postexposure serum) of proteins from *P. multocida* grown at 32°C for 18 h, shifted to 42°C for 10 min, labelled for 15 min at 42°C, and then separated by two-dimensional polyacrylamide gel electrophoresis. Spots A through D represent immunoreactive proteins.

reactive proteins, represented by spots B (60 kDa) and D (35 kDa), did not seem to be affected by the increase in temperature, although the intensity of spot B appeared greater at 42°C than at 32°C. After an hour at 42°C, the intensity of spots A, C, and D were greatly reduced, whereas spot B intensity was equal to that after 10 min of heat shock (data not shown). This observation implies that the protein represented by spot B may also be a heat shock protein. On the other hand, proteins corresponding to spots A, B, C, and D were not detected when proteins extracted from *P. multocida* grown at 32°C were probed with either immune serum or naive serum (data not shown).

We have shown that at an increased temperature *P. multocida* produces heat shock proteins in vitro that are typical of the heat shock responses described for other bacteria (2, 4, 7). In addition, the heat shock proteins produced in *P. multocida* are of the appropriate size for the previously described families of heat shock proteins (HSP90, HSP70, HSP60, and small heat shock proteins) (3). Our data also show that turkeys that have been exposed to *P. multocida* produce antibodies specific for proteins produced by heat-shocked bacteria, implying that some of these proteins are made in vivo. While it is tempting to conclude that *P. multocida* makes increased amounts of heat shock proteins in vivo, our data do not definitively establish this. That is, it is difficult to determine whether antibodies specific for the heat shock proteins were induced by proteins made in basal amounts or by proteins made in increased amounts induced by a step up in temperature.

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