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Possible Association of GroES and Antigen 85 Proteins with Heat Resistance of *Mycobacterium paratuberculosis*

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Conflicting reports on the heat resistance of *Mycobacterium paratuberculosis* prompted an examination of the effect of culture medium on this property of the organism. *M. paratuberculosis* was cultured in three types of media (fatty acid-containing medium 7H9-OADC (oleic acid-albumin-dextrose-catalase supplement) and glycerol-containing media WR-GD and 7H9-GD [glycerol-dextrose supplement]) at pH 6.0. *M. paratuberculosis* grown under these three culture conditions was then tested for heat resistance in distilled water at 65°C. Soluble proteins and mycolic acids of *M. paratuberculosis* were evaluated by two-dimensional electrophoresis (2-DE) and thin-layer chromatography (TLC), respectively. The type of culture medium used significantly affected the heat resistance of *M. paratuberculosis*. The decimal reduction times at 65°C ($D_{65°C}$ values; times required to reduce the concentration of bacteria by a factor of 10 at 65°C) for *M. paratuberculosis* strains grown in 7H9-OADC were significantly higher than those for the organisms grown in WR-GD medium ($P < 0.01$). When the glycerol-dextrose supplement of WR was substituted for the fatty acid supplement (OADC) in 7H9 medium (resulting in 7H9-GD), the $D_{65°C}$ value was significantly lower than that for the organism grown in 7H9-OADC medium ($P = 0.022$) but higher than that when it was cultured in WR-GD medium ($P = 0.005$). Proteomic analysis by 2-DE of soluble proteins extracted from *M. paratuberculosis* grown without heat stress in the three media (7H9-OADC, 7H9-GD, and WR-GD) revealed that seven proteins were more highly expressed in 7H9-OADC medium than in the other two media. When the seven proteins were subjected to matrix-assisted laser desorption ionization-mass spectrometric analysis, four of the seven protein spots were unidentifiable. The other three proteins were identified as GroES heat shock protein, alpha antigen, and antigen 85 complex B (Ag85B; fibronectin-binding protein). These proteins may be associated with the heat resistance of *M. paratuberculosis*. Alpha antigen and Ag85B are both trehalose mycolyltransferases involved in mycobacterial cell wall assembly. TLC revealed that 7H9-OADC medium supported production of more trehalose dimycolates and cell wall-bound mycolic acids than did WR-GD medium. The present study shows that in vitro culture conditions significantly affect heat resistance, cell wall synthesis, and protein expression of *M. paratuberculosis* and emphasize the importance of culture conditions on in vitro and ex vivo studies to estimate heat resistance.

Mycobacterium paratuberculosis (also known as *Mycobacterium avium* subsp. *paratuberculosis*) is the cause of Johne's disease in animals, in particular dairy cattle (14). Numerous studies report a significant association of this pathogen with the chronic inflammatory bowel disease of humans called Crohn's disease (10, 17, 21, 45, 50). Consequently, *M. paratuberculosis* has come under scrutiny as a potential food-borne pathogen of dairy products (16). For this reason, studies of *M. paratuberculosis* heat resistance, specifically survival in the face of milk pasteurization, are important (12, 33, 38).

Results from previous assessments of heat resistance for *M. paratuberculosis* have varied. Hope et al. (30) reported that *M. paratuberculosis* was eliminated by pasteurization since no viable organisms were recovered after heat treating 10^4 cells/ml at 72°C for 25 s. Grant et al. (27, 28) and Sung and Collins (54) found that not all *M. paratuberculosis* organisms were eliminated by the high-temperature short-time (71°C for 15 s) method of pasteurization. Lund et al. (38) and Klijn et al. (33) in their reviews of the topic attributed the different results found in these studies to dissimilar experimental conditions. Of particular importance were methods for managing clumps of *M. paratuberculosis*, application of heat, and recovery of heat-injured organisms.

The reviewers did not mention an additional factor of importance for heat resistance research, i.e., in vitro culture conditions for *M. paratuberculosis*. Previous work from our laboratory showed that culture conditions for *M. paratuberculosis* cell production can significantly affect its ability to withstand exposure to low pH (56).

In the present report we extend studies on culture media to evaluate their influence on the heat resistance of *M. paratuberculosis*. We employed proteomic approaches to identify proteins associated with higher heat resistance of *M. paratuberculosis* that are expressed under specific culture conditions. We evaluated the effect of culture medium on proteins associated with mycolic acid-containing cell wall components.

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MATERIALS AND METHODS

Culture media. Three types of liquid culture media were used in this study: oleic acid ($C_{17}H_{33}COOH$; fatty acid)-supplemented Middlebrook 7H9-OADC (oleic acid-albumin-dextrose-catalase; Difco, Detroit, Mich.), glycerol-supple-

mented WR-GD (glycerol-dextrose), and 7H9-GD medium enriched with 0.0002% (wt/vol) mycobactin J (Allied Monitor Inc., Fayette, Mo.). The detailed composition of each medium was described previously.

***M. paratuberculosis* strains.** Two bovine *M. paratuberculosis* strains were used in this study: a clinical strain (JTC303) and a reference strain (ATCC 19698). The clinical strain was isolated in our laboratory from a Holstein cow with clinical paratuberculosis. Its identity was verified by PCR for IS*900* and mycobactin dependency.

The *M. paratuberculosis* seed lots were prepared as previously described. That is, *M. paratuberculosis* strains were cultured at 37°C for 4 months in 7H9-OADC, WR-GD, and 7H9-GD media adjusted at pH 6.0. Then, the *M. paratuberculosis* cultures were centrifuged and washed three times with 30 ml of phosphate-buffered saline (PBS; 10 mM, pH 6.8). The suspensions were homogenized with an overhead stirrer (Wheaton Instruments, Milville, N.J.) for 4 min on ice to break up large clumps of *M. paratuberculosis* cells, enumerated by a radiometric culture method (BACTEC) described in our previous publications (54–56), and stored in multiple small aliquots at −70°C as the inoculum.

***M. paratuberculosis* cultures.** The *M. paratuberculosis* seed lots for both strains were cultured in tissue culture flasks (75-cm^2 canted neck; Costar, Cambridge, Mass.) containing 35 ml of 7H9-OADC, WR-GD, and 7H9-GD media at pH 6.0. Based on the growth curves obtained previously (56), organisms were harvested from flasks at the early stationary phase of growth for each medium. The stationary-phase cultures of *M. paratuberculosis* strains were washed, homogenized, and enumerated as explained above. These stationary-phase cultures were stored in multiple small aliquots at −70°C for subsequent heat treatment trials as explained below.

Heat treatment. *M. paratuberculosis* cells were suspended in Wheaton vials (12 by 35 mm; Kimble Glass Co., Vineland, N.J.) in a total volume of 1.5 ml of distilled water as a menstruum preheated for 30 min in a water bath (5L-M; Fisher Scientific Co., Medford, Mass.) to the reaction temperature of 65°C. Each vial was sealed and immersed in the water bath. The menstruum in the vials was kept 4 cm below the water level in the bath. The temperature was monitored at all times with a mercury-filled thermometer (Fisher Scientific, Pittsburgh, Pa.) and was maintained ±0.5°C. The final concentration of *M. paratuberculosis* was 10^5 to 10^6 cells/ml. After being heated for 0, 60, 120, 180, 240, and 300 s at 65°C, vials were removed in triplicate at each holding time from the water bath and immediately chilled by immersion in ice water. Viable *M. paratuberculosis* cells from each vial were enumerated by the radiometric culture method (BACTEC) (54–56).

***D* value.** The decimal reduction times at 65°C ($D_{65°C}$ values; times required to reduce the concentration of bacteria by a factor of 10 at 65°C) were calculated from the slope of the best-fit line graphically determined by plotting the $\log_{10}$ of the number of viable *M. paratuberculosis* cells/ml versus heating time at 65°C, as previously described (54–56). The best-fit lines were calculated by a linear regression analysis (Prism, version 3.03; GraphPad Software, Inc., San Diego, Calif.) and Minitab (State College, Pa.) regression analysis software (release 13).

Soluble proteins extracted from the cell pellet. Early-stationary-phase *M. paratuberculosis* cells grown in the three culture media at pH 6.0 were pelleted and washed three times by centrifugation at 30,000 × *g* for 10 min with 30 ml of PBS (10 mM, pH 6.8) containing 0.05% (vol/vol) protease inhibitor cocktail (Sigma, St. Louis, Mo.). The cell pellets were disrupted with glass beads (glass perlen, 0.10 to 0.11 mm) and a cell homogenizer (model MSK; Braun Instruments, Burlingame, Calif.). Homogenization was performed for 5 min with discontinuous cooling by liquid CO_2. After homogenization, the glass beads were recovered by centrifugation at 1,500 × *g* for 10 min. The homogenate was then transferred to another sterile tube and centrifuged at 30,000 × *g* for 1 h at 4°C. The supernatant of soluble proteins was concentrated with Centriprep centrifuge PM-3 concentrators (Amicon, Beverly, Mass.) and sterilized by Millipore filters (0.2-μm pore size). The protein concentration was determined by the protocol of bicinchoninic acid assay (48, 52) with a protein assay kit (Pierce, Rockford, Ill.). The soluble protein extracts were then stored at −70°C.

2-DE. Two-dimensional electrophoresis (2-DE) for the *M. paratuberculosis* soluble proteins was performed according to the method of O'Farrell (46) by Kendrick Labs, Inc. (Madison, Wis.) as follows. Isoelectric focusing was carried out in glass tubes having an inner diameter of 2.0 mm with 2% pH 4 to 8 ampholines (Gallard-Schlesinger Industries, Inc., Garden City, N.Y.) for 9,600 V-h. One microgram of an internal standard, tropomyosin protein, with a lower spot of 33 kDa and pI 5.2, was added to the soluble proteins (200 μg).

After soaking for 10 min in equilibration buffer (10% [vol/vol] glycerol, 2.3% [wt/vol]) sodium dodecyl sulfate [SDS], 0.0625 M Tris, pH 6.8), each tube gel was sealed to the top of a staking gel that overlay 10% acrylamide slab gels (0.75 mm thick). SDS slab gel electrophoresis was carried out for about 4 h at 12.5 mA/gel. The protein spots were then visualized by Coomassie brilliant blue R250 staining. The standard proteins (Sigma) were added to the agarose that sealed the tube gel to the slab gel.

Documentation and analysis of 2-DE gels. To obtain well-defined digital images for the protein spots, a high-resolution transparency scanner (PowerLook III; UMAX Technologies Inc., Dallas, Tex.) was used with the Coomassie brilliant blue-stained 2-DE gels. The digital images were analyzed with computer software (Phoretix 2D Advanced, version 6.01; Nonlinear Dynamics, Durham, N.C.) for spot detection, background subtraction, spot matching, spot volume normalization, and gel comparison. The volume (intensity) of each spot was divided by the total volume of all of the spots in the gel after subtracting background intensity and was multiplied by 100, a scaling factor. Thus, the spot volume for each protein spot in a gel represents expression percentages calculated on the basis of spot intensity.

Based on the volumes, protein spots were compared and several were selected for identification by mass fingerprinting as explained below.

In-gel digestion of protein spots. The protein spots of interest were excised from the dried 2-DE gels, placed in clean, pretrypsinized Eppendorf tubes, and rehydrated with distilled water (50 μl) by Kendrick Labs, Inc. When the gel pieces were completely rehydrated, the water was discarded, and the gel pieces were destained by washing with 50 mM Tris-HCl, pH 8.5-50% acetonitrile three times for 20 min and then washed once with 100% acetonitrile for 1 min. The gel pieces were dried completely in a vacuum centrifuge and rehydrated with 0.06 μg of modified trypsin (Roche Molecular Biochemicals; sequencing grade) in 12 μl of 25 mM Tris-HCl, pH 8.5. The tubes containing the gel pieces were placed in a heating block at 32°C and left overnight.

Peptide mass fingerprinting by MALDI-MS. The in-gel-digested peptides were redissolved in 3 μl of matrix solution (10 mg of 4-hydroxy-α-cyanocinnamic acid/ml in 50% acetonitrile–0.1% trifluoroacetic acid) with angiotensin and bovine insulin added as internal calibration standards, and 0.6 μl of this solution was spotted onto a sample plate, dried, and washed twice with 2 μl of distilled water. Matrix-assisted laser desorption ionization-mass spectrometric (MALDI-MS) analysis was performed in the linear mode using a PerSeptive Voyager DE-RP mass spectrometer in the Protein Core Facility at Columbia University, New York, N.Y. The National Center for Biotechnology Information (NCBI) database was searched with the resulting peptide masses by using the ProFound search program (ProteoMetrics).

Preparation of TDM-containing lipids. *M. paratuberculosis* JTC303 was grown in 7H9-OADC and WR-GD media at pH 6.0, collected at early stationary phase, washed three times with 30 ml of PBS (10 mM, pH 6.8), and homogenized as explained above. The trehalose dimycolate (TDM) was extracted and analyzed by a procedure previously described (57). The *M. paratuberculosis* JTC303 cells were washed again with ethanol, suspended in acetone, filtered, and air dried. These cells (100 mg in dry weight) were suspended with 7 ml of chloroform-methanol (2:1), left overnight, and centrifuged at 4,800 × *g* for 10 min. The supernatant was recovered, and the pellet was reextracted five times with 7 ml of chloroform-methanol (2:1). This step included mixing well by vortexing for 1 min, sonicating in a sonicator water bath for 15 min, and centrifuging at 4,800 × *g* for 10 min. The cell pellet was saved as the source of cell wall-bound mycolic acids (see below). The chloroform-methanol extract was pooled, filtered, and dried with a steam of nitrogen in a warm-water bath (analytical evaporator, Meyer N-EVAP model 12; Organomation Associates Inc., Northborough, Mass.). The dried residues were dissolved with 2 ml of chloroform-methanol-water (2:3:1) and applied to Sep-Pak Accell Plus QMA cartridges (Water Corporation, Milford, Mass.) preconditioned with the same solvent. The effluent containing neutral lipid fractions was collected and dried. The neutral lipid fraction was dissolved in 2.0 ml of chloroform and applied to preconditioned Sep-Pak silica cartridges (Water Corporation), and the effluent was discarded. The TDM-containing lipids were eluted by passing 2 ml of chloroform-methanol at 9:1 and 2 ml of chloroform-methanol at 85:15 through the cartridges. The pooled effluents were dried and weighed. This fraction containing TDM was analyzed by thin-layer chromatography (TLC; see below).

Preparation of cell wall-bound mycolic acids. The cell wall-bound mycolic acids were extracted and analyzed by a procedure previously described (57). A portion of the delipidated cells was suspended in 1% (wt/vol) SDS, heated in boiling water bath for 60 min, and centrifuged at 4,800 × *g* for 10 min. This SDS treatment was repeated three times, and each time the supernatant was discarded. The pellet containing the cell wall skeleton was washed with ethanol and acetone and then dried and weighed. This preparation was suspended with 2.0 ml of 2 M KOH, incubated at 56°C for 60 min, and cooled at room temperature. Then, the cell wall skeleton suspensions were acidified (around pH 1.0) with 6 M HCl, mixed with 5 ml of chloroform-methanol (2:1), and centrifuged. The upper aqueous layer was discarded. The lower organic layer containing free mycolic acids was collected, filtered, dried, and weighed.

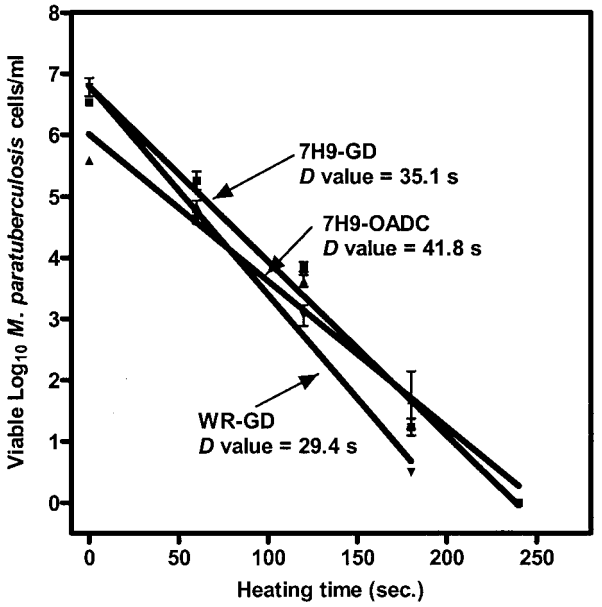

FIG. 1. Thermal death curves for *M. paratuberculosis* JTC303 at 65°C after culture in 7H9-OADC, WR-GD, and 7H9-GD media at pH 6.0. Error bars represent standard deviations ($n = 3$).

TLC. For analytical TLC, silica gel GHL plates (250 μm; Analtech Inc., Newark, Del.) were used. The solvent system of chloroform-methanol-concentrated ammonium hydroxide (40:10:1) was used for the separation of TDM. The free mycolic acids were methylated with diazomethane, and the methyl esters were analyzed by TLC using the solvent system of petroleum ether-diethyl ether (7:1). For visualization of the bands, TLC plates were sprayed lightly with 0.6% (wt/vol) potassium dichromate in sulfuric acid (55% [wt/vol]) and charred.

Statistical analysis. Linear regressions of inactivation curves for $D_{65°C}$ were based on the concepts presented by Chatterjee and Price (11) and Draper and Smith (20). Differences among slopes of inactivation curves were analyzed with Prism software (GraphPad Software, Inc.) as previously described (54–56). Differences between the amounts of cell wall-bound mycolic acids derived from cell wall skeletons of *M. paratuberculosis* cells grown in 7H9-OADC and WR-GD media were analyzed by the Mann-Whitney test using Instat software (version 3.0; GraphPad Software, Inc.). P values of <0.05 were considered significant.

RESULTS

Heat resistance of *M. paratuberculosis*. Figure 1 shows representative thermal death curves, displaying data for *M. paratuberculosis* strain JTC 303 cultured in 7H9-OADC, WR-GD, and 7H9-GD at pH 6.0 and tested for thermal resistance at 65°C. All thermal death curves for *M. paratuberculosis* strains cultured in 7H9-OADC, WR-GD, and 7H9-GD media were linear ($r^2 > 0.90$) with narrow 95% confidence intervals (data not shown). The $D_{65°C}$ values for *M. paratuberculosis* strains from the three media were determined from the slopes of thermal death curves and are shown in Table 1.

Heat resistance of the clinical (JTC303) and the laboratory-adapted reference (ATCC 19698) *M. paratuberculosis* strains was significantly different when the strains were cultured in 7H9-OADC medium. That is, the $D_{65°C}$ value for the JTC303 strain was 41.8 s when the strain was cultured in 7H9-OADC medium, while that for strain ATCC 19698 was 47.6 s ($P = 0.041$). However, when the clinical and reference *M. paratuberculosis* strains were cultured in WR-GD medium, the $D_{65°C}$ values for them were not significantly different ($P = 0.3565$).

TABLE 1. $D_{65°C}$ values for *M. paratuberculosis* strains grown in 7H9-OADC, WR-GD, and 7H9-GD media at pH 6.0

Culture medium	$D_{65°C}$ value(s) for strain:	
	JTC303	ATCC 19698
7H9-OADC	41.8[ab]	47.6[b]
WR-GD	29.4	32.4
7H9-GD	35.1[c]	NT[d]

[a] Significantly different from the *D* value for ATCC 19698 cultured in 7H9-OADC medium.
[b] Significantly different from *D* values for the same strain cultured in glycerol-dextrose-supplemented media (WR-GD and 7H9-GD).
[c] Significantly different from *D* values for the same strain cultured in WR-GD medium.
[d] NT, not tested.

The type of culture medium also had a significant effect on *M. paratuberculosis* heat resistance. Greater resistance was observed for *M. paratuberculosis* strains grown in 7H9-OADC than in WR-GD or 7H9-GD (Table 1). Specifically, $D_{65°C}$ values were significantly higher for both the clinical and reference strains cultured in 7H9-OADC than those for WR-GD medium-grown organisms ($P < 0.01$). The $D_{65°C}$ value for *M. paratuberculosis* strain JTC303 was significantly lower when the organism was cultured in 7H9-GD medium than when it was cultured in 7H9-OADC medium ($P = 0.022$). Heat resistance for the organism cultured in 7H9-GD was greater than that when it was cultured in WR-GD medium, as shown by a higher $D_{65°C}$ value in 7H9-GD medium than in WR-GD medium ($P = 0.005$) for *M. paratuberculosis* strain JTC303 (Table 1).

2-DE protein profiles. The 2-DE soluble-protein profiles for early-stationary-phase *M. paratuberculosis* strain JTC303 cultured in 7H9-OADC, WR-GD, and 7H9-GD at pH 6.0 revealed that protein expression differed depending on the type of medium used. WR-GD medium-grown *M. paratuberculosis* cells yielded the highest number of protein spots (452), followed by cells grown in 7H9-OADC (346) and 7H9-GD (314).

When comparison of protein spot volumes (expression percentages, as explained in Materials and Methods) was made with WR-GD medium as a baseline, it was found that 56 (16.2%) spots were increased more than twofold for organisms cultured in 7H9-OADC versus WR-GD and that 53 (16.9%) protein spots were increased more than twofold for organisms grown in 7H9-GD versus WR-GD. A twofold or greater reduction in protein spot volume was seen for 46 (13.3%) protein spots from 7H9-OADC-grown cells and for 33 (10.5%) protein spots for 7H9-GD-grown cells compared to the WR-GD protein profile (Fig. 2).

Analysis and identification of protein spots. Seven 7H9-OADC medium protein spots with higher intensity than that observed from WR-GD-grown organisms were selected for further analysis (Fig. 3) since they were strongly associated with the alternation in *M. paratuberculosis* heat resistance. Of low molecular mass, these were proteins of 12 kDa with pI values of 5.13 and 5.30 (CS12-5.13 and CS12-5.30), 14 kDa with a pI value of 5.15 (CS14-5.15), 19 kDa with pI values of 5.29 and 5.96 (CS19-5.29 and CS19-5.96), and 29 kDa with pI values of 5.37 and 5.51 (CS29-5.37 and CS29-5.51).

While the expression of these seven protein spots was higher when *M. paratuberculosis* was cultured in 7H9-OADC than

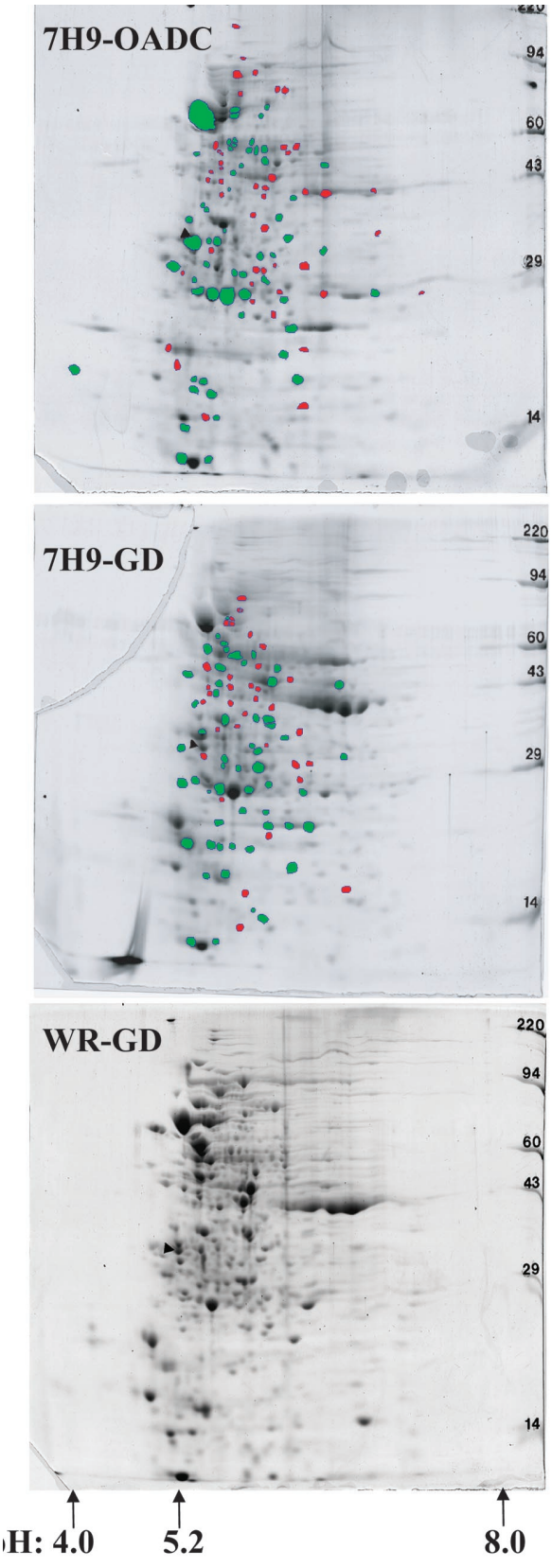

FIG. 2. Comparison of protein spot volumes for *M. paratuberculosis* JTC303 cultured in 7H9-OADC versus WR-GD medium and 7H9-GD versus WR-GD medium. The protein spot volumes in 2-DE gels of *M. paratuberculosis* cultured in 7H9-OADC and 7H9-GD media at pH 6.0 were obtained as described in Materials and Methods and compared with protein spot volumes for the organism cultured in WR-GD at pH 6.0. Green spots, volume increased more than twofold; red spots, volume decreased more than twofold.

when it was cultured in WR-GD medium, the expression levels for these spots were decreased when the comparison was made between 7H9-OADC and 7H9-GD. This was especially true for the expression levels for protein spots CS14-5.15 and CS19-5.29. Their levels were decreased to approximately the same levels as those seen in WR-GD medium (Fig. 3A and B).

To identify the seven protein spots, they were subjected to MALDI-MS analysis and their peptide masses were compared with those of known *M. paratuberculosis* proteins in the NCBI database (Table 2). Spot CS12-5.13 was identified as a 10-kDa chaperonin *M. paratuberculosis* protein (also called GroES or HSP10) (15, 23), and spot CS29-5.51 was confirmed to be fibronectin-binding antigen 85 complex B (also called Ag85B) (18). Spot CS29-5.37 was identified as an *M. avium* alpha antigen protein. The 12- (CS12-5.30), 14- (CS14-5.15), and 19-kDa protein spots (CS19-5.29 and CS19-5.96) did not match any previously identified mycobacteria proteins in the NCBI database and thus can be considered novel proteins.

Analysis of TDM-containing lipids and cell wall-bound mycolic acids. Figure 4 shows thin-layer chromatograms of TDM-containing lipids (A) and cell wall-bound mycolic acids (B) of *M. paratuberculosis* JTC303 when cultured in 7H9-OADC medium and WR-GD medium at pH 6.0. When strain JTC303 was cultured in 7H9-OADC, 0.93 ± 0.06 mg of TDM-containing lipids was obtained, lower than the amount obtained when the strain was grown in WR-GD medium (1.48 ± 0.13 mg). However, TLC clearly showed that more TDM molecules were generated when *M. paratuberculosis* JTC303 was cultured in 7H9-OADC medium than when it was cultured in WR-GD medium (A). Nonpolar lipids may be responsible for the higher dry weight for TDM-containing lipids of *M. paratuberculosis* JTC303 cultured in WR-GD medium.

The 7H9-OADC medium supported greater production of cell wall-bound mycolic acids of *M. paratuberculosis* than the WR-GD medium (Table 3 and Fig. 4B). When the cell wall skeletons were prepared from *M. paratuberculosis* JTC303 grown in 7H9-OADC and WR-GD media, 20.2% ± 0.5% and 17.6% ± 1.2% of mycolic acids were derived, respectively. This difference was statistically significant ($P = 0.0238$) and was verified by checking the purity of mycolates seen on TLC (Fig. 4B). The mycolic acid subclass patterns (α, keto, and ω-carboxy mycolates) were the same whether derived from *M. paratuberculosis* JTC303 grown in 7H9-OADC or WR-GD medium. (The identification of the ω-carboxy mycolates was based on previous reports on mycolic acid patterns for *M. paratuberculosis* [42, 43].)

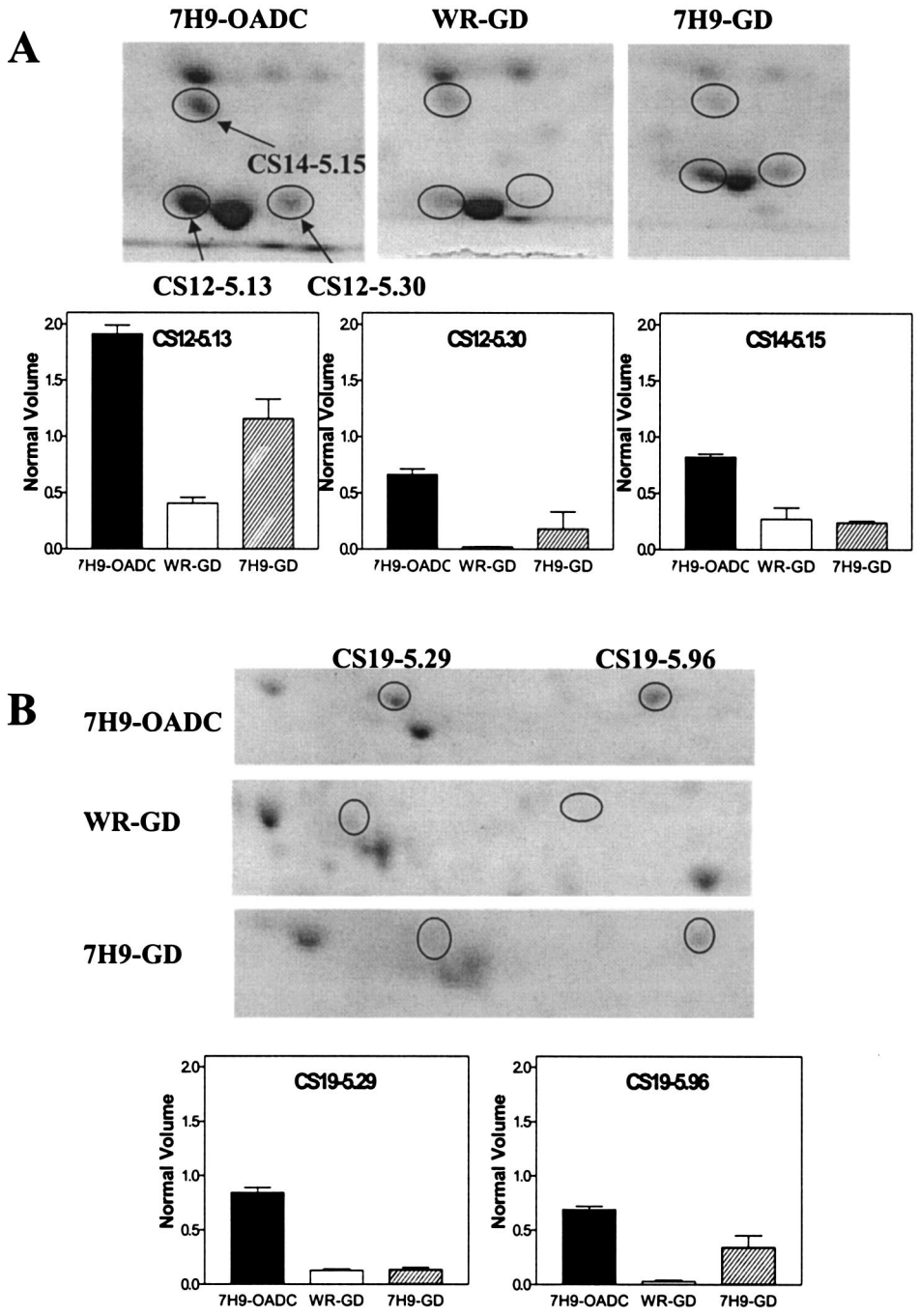

FIG. 3. The two-dimensional gel regions for spot volume comparisons of 12- (CS12-5.13 and CS12-5.30) and 14- (CS14-5.15) (A), 19- (CS19-5.29 and CS19-5.96) (B), and 29-kDa protein spots (CS29-5.37 and CS29-5.51) (C) of *M. paratuberculosis* JTC303 when cultured in 7H9-OADC, WR-GD, and 7H9-GD media at pH 6.0. The spot volumes for the protein spots in 2-DE gels of *M. paratuberculosis* were obtained and plotted as described in Materials and Methods. Error bars represent standard errors of the means, which were obtained from two individual gel images.

DISCUSSION

This is the first study reporting that in vitro culture medium significantly affects heat resistance, cell wall synthesis, and protein expression of *M. paratuberculosis*. Mycobacteria are cultivated for diagnostic and research purposes primarily in egg yolk-based solid media, such as Löwenstein-Jensen medium and Herrold's medium, and in liquid media, such as Middlebrook 7H9, Watson-Reid, Sauton, and Dubos media. These media were developed with the primary goal of achieving rapid growth for faster diagnosis of tuberculosis (60). Culture media

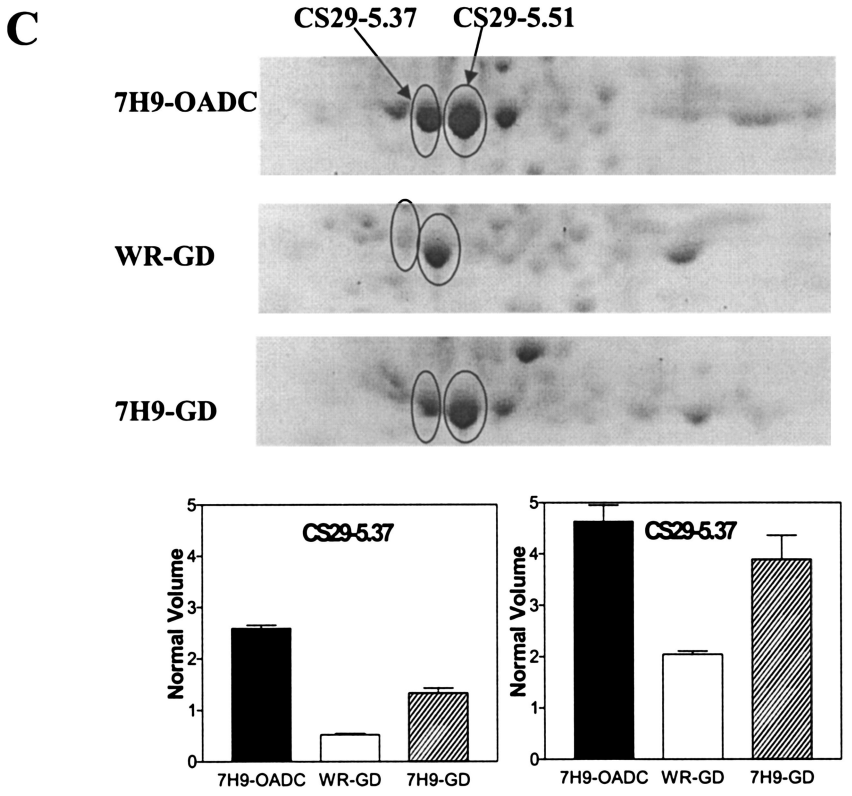

FIG. 3—*Continued*.

for *M. paratuberculosis* were adapted from the media developed for rapid cultivation of *Mycobacterium tuberculosis* bacilli (40, 41, 62). No thought was given to any particular requirements or the specific physiology of *M. paratuberculosis* other than its in vitro growth requirement for mycobactin. In this study, we report that *M. paratuberculosis* grown in a fatty acid-containing medium possessed a degree of heat resistance, protein expression profile, and cell wall mycolic acid pattern significantly different from those when the organism was grown in glycerol-containing media.

The type of menstruum in which *M. paratuberculosis* was suspended did not significantly influence the *D* values for the organism. The $D_{65°C}$ value (41.8 s when cultured in 7H9-OADC at pH 6.0) in distilled water for *M. paratuberculosis* clinical strain JTC303 was not significantly different from that (44.2 s in lactate solution) previously reported (54). The *D* values we previously reported at different temperatures (65, 68, and 71°C) for *M. paratuberculosis* clinical strains in lactate solution (50 mM) versus milk also were not significantly different (54). Hence, clinical *M. paratuberculosis* strains showed the same heat resistance patterns whether suspended in lactate solution, milk, or distilled water.

Examining heat resistance of only two strains of *M. paratuberculosis* cannot provide a full investigation of strain variation. However, we and others previously reported that both heat and acid resistance responses for the laboratory-adapted *M. paratuberculosis* ATCC 19698 strain were significantly different from those for the clinical strains (low passage) when the organisms were cultured in 7H9-OADC medium (12, 54, 56). In the present study, the $D_{65°C}$ values for the bovine clinical strain and the reference strain were also significantly different when strains were cultured in 7H9-OADC. This did not hold true for organisms grown in WR-GD medium, however (Table 1). These data indicate that detection of differences in heat resistance between clinical and laboratory-adapted strains may be due to choice of culture media. If the same pattern of strain difference in 7H9-OADC medium holds true at other temperatures, it has significant implications for studies assessing the ability of milk pasteurization or other thermal processes designed to kill this pathogen.

The type of culture medium used significantly influenced the heat resistance of *M. paratuberculosis*. Growth in the fatty acid medium (7H9-OADC) resulted in higher $D_{65°C}$ values for *M. paratuberculosis* than did growth in the glycerol medium (WR-GD). The $D_{65°C}$ values for 7H9-OADC-grown *M. paratuberculosis* were significantly lowered when the organism was cultured in 7H9-GD medium. These findings were also observed in our previous report assessing acid resistance of *M. paratuberculosis* (56). In summary, fatty acid medium (7H9-OADC) generated *M. paratuberculosis* cells with greater heat and acid resistance than glycerol-containing media (WR-GD and 7H9-GD) and glycerol appeared to be the medium component most affecting heat and acid resistance of the organism.

The $D_{65°C}$ values for *M. paratuberculosis* cultured in 7H9-OADC medium were higher than those previously published for other pathogenic bacteria such as *Listeria monocytogenes* (6.0 to 7.3 s) (7, 19), *Salmonella enterica* serovar Typhimurium (6.7 s for $D_{62.8°C}$) (8), and *M. avium* (31.6 s) (54). However, the

TABLE 2. 2-DE protein spots identified by peptide mass fingerprinting using MALDI-MS

Spot	Observed molecular mass (kDa)	Observed pI	Theoretical molecular mass (kDa)	Theoretical pI	Identified protein	NCBI accession no.	Mass		Residues	Matched peptide sequence
							Measured[a]	Computed		
CS12-5.13	12.0	5.13	10.73	4.7	10-kDa chaperonin (GroES protein) (*M. paratuberculosis*)	AAD23277	1,427.67	1,427.577	81–92	YNGEEYLILSAR
							1,523.74	2,523.709	36–50	EKPQEGTVVAVGPGR
							1,791.92	1,792.019	58–73	RIPLDVSEGDTVIYSK
							2,311.40	2,311.493	36–57	EKPQEGTVVAVGPGRWDDDGAK
							2,342.70	2,342.629	13–35	ILVQANEAETTTASGLVIPDTAK
							2,385.06	2,384.665	59–80	IPLDVSEGDTVIVSKYGGTEIK
CS29-5.37	29.0	5.37	34.92	5.3	Alpha antigen (*M. avium*)	S32773	1,674.63	1,674.814	216–230	ADAMWGPSSDPAWQR
							1,798.96	1,798.976	231–246	NDPSLHIPELVGHNTR
							1,986.05	1,986.208	137–153	WETFLTSELPAYLASNK
							2,078.58	2,078.312	64–83	VQFQSGGNGSPAVYLLDGLR
							2,767.83	2,768.096	130–153	AGCSTYKWETFLTSELPSYLASNK
							3,143.49	3,143.551	247–274	LWLYCGNGTPSEVGGANMPAEFLENFVR
CS29-5.51	29.0	5.51	34.89	5.3	Fibronectin-binding Ag85B (*M. paratuberculosis*)	AF219121	1,674.93	1,674.814	216–230	ADAMWGPSSDPAWQR
							1,799.09	1,798.976	231–246	NDPSLHIPELVGHNTR
							1,986.39	1,986.208	137–153	WETFLTSELPAYLASNK
							2,078.68	2,078.312	64–83	VQFQSGGNGSPAVYLLDGLR
							2,767.83	2,768.096	130–153	AGCSTYKWETFLTSELPSYLASNK
							3,143.31	3,143.551	247–274	LWVYCGNGTPSELGGANMPAEFLENFVR

[a] Peptide mass/charge (m/z) value. Peptides were singly charged by protons (MH^+).

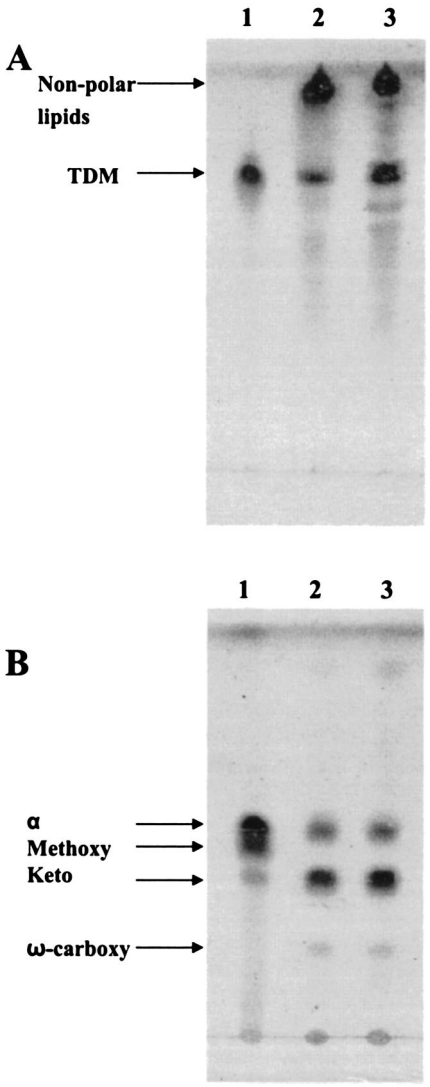

FIG. 4. TLC of TDM-containing lipids (A) and cell wall-bound mycolic acids as methyl esters (B) for *M. paratuberculosis* JTC303 cultured in WR-GD and 7H9-OADC media at pH 6.0. The amounts of TDM-containing lipids and cell wall methyl mycolates applied to the TLC plates were 50 and 15 μg, respectively. The standards for TDM and methyl mycolates were obtained from late-log-phase *M. tuberculosis* H37Ra cultured in 7H9-OADC medium. Lane 1, standard; lane 2, samples derived from WR-GD; lane 3, samples derived from 7H9-OADC.

$D_{65°C}$ values for *M. paratuberculosis* cultured in WR-GD and 7H9-GD media were lower or the same as those for *M. avium*.

Among the culture media we used, the WR-GD medium produced the most thermally sensitive organism and supported production of the largest number of *M. paratuberculosis* proteins based on 2-DE protein profile analysis. Conversely, 7H9-OADC-grown organisms showed the highest $D_{65°C}$ values but produced the smallest number of proteins. Thus, to identify proteins that may be associated with heat resistance, we focused on *M. paratuberculosis* protein spots more highly expressed in 7H9-OADC medium than in WR-GD medium but with reduced expression in 7H9-GD medium.

We selected seven protein spots in 7H9-OADC medium that showed high intensities and expression patterns strongly associated with the observed change in heat resistance of *M. paratuberculosis*. Among the seven protein spots, two protein spots were identified as *M. paratuberculosis* proteins (GroES heat shock protein and Ag85B protein) and one was found to be an *M. avium* protein (alpha antigen) when subjected to MALDI-MS analysis (Table 2). We were fortunate to identify two *M. paratuberculosis* proteins since there are only 175 proteins for *M. paratuberculosis* in NCBI database (*M. tuberculosis* has about 7,000 to 8,000 identified proteins (http://www.ncbi.nlm.nih.gov/Taxonomy) (6, 61).

TABLE 3. Cell wall-bound mycolic acids derived from cell wall skeleton preparations of *M. paratuberculosis* JTC303 grown in 7H9-OADC and WR-GD media at pH 6.0

Culture medium	Cell wall skeleton dry wt (mg)	Cell wall-bound mycolic acids	
		Dry wt (mg)	%
7H9-OADC	49.5	9.8	20.2 ± 0.5
	32.5	6.5	
	32.3	6.7	
WR-GD	50.2	9.5	17.6 ± 1.2
	50.1	8.0	
	48.4	8.5	
	44.8	8.4	
	40.0	7.3	
	38.0	6.2	

The GroES heat shock protein has been found in culture filtrate, cytosol, and cell walls of *M. tuberculosis* and *Mycobacterium leprae* (1, 4, 39, 47, 49, 58) and is a well-documented T-cell antigen (13, 32, 36). The molecular mechanisms of heat resistance due to GroES in mycobacteria are unknown. However, it has been shown that the protein contributes to protein folding, repair, and degradation by inducing expression of a sigma factor (σ^{32}) in *Escherichia coli* (3, 26, 29, 35, 51, 53, 65). It is likely that the same functions occur in mycobacteria, functions that may contribute to heat resistance for 7H9-OADC-grown *M. paratuberculosis*. That is, the greater abundance of GroES proteins in 7H9-OADC-grown *M. paratuberculosis* may have been responsible for protecting cells from heat through prompt mending of denatured or misfolded proteins.

Ag85B and alpha antigen belong to the Ag85 complex, consisting of Ag85A (32 kDa), Ag85B (30 kDa), and Ag85C (32.5 kDa) (63). This Ag85 complex has been identified as a major secreted protein of actively replicating *M. tuberculosis* (63). It is also closely associated with the cytosol and the cell wall of mycobacteria since extracts of disrupted cells, prepared by sonication, contain the complex (44, 64).

The Ag85 complex comprises trehalose mycolyltransferases involved in the final stages of mycobacterial cell wall assembly (2, 5, 31, 34, 63). That is, the Ag85 complex transfers mycolic acids from trehalose monomycolate to form the predominant cell wall components, TDM (cord factor) and arabinogalactan-mycolate (AGM). It has been proposed that the mycolic acids in the forms of TDM and AGM in mycobacterial cell walls act as a protective physical barrier with low fluidity and permeability for hydrophobic and hydrophilic molecules, such as antibiotics (22, 24, 25, 31, 37, 59).

Considering the abundance of trehalose mycolyltransferases (Ag85B and alpha antigen) in 7H9-OADC-grown *M. paratuberculosis* and the important role of the enzyme for cell wall formation in mycobacteria, we compared the production of mycolic acid-containing components (TDM and cell wall-bound mycolic acids) of the organisms when cultured in 7H9-OADC medium with that when they were cultured in WR-GD medium. The 7H9-OADC-grown *M. paratuberculosis* contained larger amounts of TDM and cell wall-bound mycolic acids than WR-GD-grown organisms (Fig. 4 and Table 3). Thus, 7H9-OADC-grown *M. paratuberculosis* may possess a greater capacity to construct a thick impervious cell wall than WR-GD-grown organisms.

Culture medium did not affect the subclass pattern of mycolic acids in *M. paratuberculosis*. That is, we found that cell wall-bound mycolic acids derived from *M. paratuberculosis* JTC303 cultured in two medium types (7H9-OADC and WR-GD) had the same α, keto, ω-carboxy mycolate subclass patterns (Fig. 4A) and were free of methoxymycolates, as shown by TLC analysis (Fig. 4B) previously reported (42, 43). Butler et al. (9) also reported that neither culture age nor medium type affected the subclass pattern of the mycolic acids for *Corynebacterium*, *Nocardia*, and *Mycobacterium* species.

In addition to the GroES and Ag85 proteins, four unidentified proteins (CS12-5.30, CS14-5.15, CS19-5.29, and CS19-5.96) were found in *M. paratuberculosis* grown in 7H9-OADC medium. Although possible functions in heat resistance cannot be predicted, the expression patterns of these proteins in 7H9-OADC, 7H9-GD, and WR-GD media strongly suggest their association with heat resistance of *M. paratuberculosis*.

In summary, heat resistance for *M. paratuberculosis* was affected by in vitro culture medium. The $D_{65°C}$ values were greater for *M. paratuberculosis* grown in 7H9-OADC than in WR-GD medium. When glycerol and dextrose were substituted for OADC in the 7H9 formulation, the $D_{65°C}$ values were reduced. This indicates that glycerol and dextrose appeared to be the medium components affecting *M. paratuberculosis* heat resistance. When *M. paratuberculosis* was cultured in 7H9-OADC, seven proteins were more strongly expressed and thus were associated with greater heat resistance. Three of the seven proteins were identified as a heat shock protein (GroES) and trehalose mycolyltransferases (Ag85B and alpha antigen) involved in mycobacterial cell wall assembly. TLC analysis showed that the higher concentrations of Ag85B and alpha antigen in *M. paratuberculosis* when cultured in 7H9-OADC medium than when cultured in WR-GD medium produced more TDM and mycolic acids with the same subclass pattern (α, keto, and ω-carboxy mycolates). The present study shows that in vitro culture conditions significantly affect heat resistance, protein expression, and cell wall formation of *M. paratuberculosis*. The results suggest possible associations of GroES and Ag85 proteins with heat resistance of *M. paratuberculosis*. These findings emphasize the impact of in vitro culture conditions on the response of *M. paratuberculosis* to physical or chemical challenges. Future thermal tolerance research for the organism in vitro or ex vivo should ensure that the significant variation introduced by different culture conditions is considered in the study design.

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