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Cloning and Expression of Portions of the 34-Kilodalton-Protein Gene of *Mycobacterium paratuberculosis*: Its Application to Serological Analysis of Johne's Disease

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Paratuberculosis (Johne's disease), an endemic mycobacteriosis of cattle that is caused by *Mycobacterium paratuberculosis*, is characterized by incoercible diarrhea and fecal shedding of bacteria. The present work aimed at developing a specific serological test for this disease. We have recently shown that a 34-kDa protein belonging to the major antigen complex A36 of *M. paratuberculosis* is immunodominant and contains epitopes specific with respect to all mycobacteria tested, including *Mycobacterium bovis* and the closely related species *Mycobacterium avium*. From a λgt11 genomic library of *M. paratuberculosis*, three portions of the gene coding for this 34-kDa protein have been isolated. Two of them expressed cross-reacting mycobacterial epitopes. One portion (in clone a362) expressed a polypeptide which cross-reacted with all tested *M. paratuberculosis* strains but not with 20 other bacteria tested, including many strains of the *M. avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* group. The occurrence at the *M. paratuberculosis* surface of epitopes corresponding to the a362 polypeptide was shown by immune electron microscopy. The recombinant a362 polypeptide was used as reagent for an enzyme-linked immunoassay for paratuberculosis. This assay correctly diagnosed all the tested blood samples from infected cattle at all stages of the disease.

Paratuberculosis (Johne's disease), a chronical enteritis produced by *Mycobacterium paratuberculosis*, affects a large proportion of ruminants in all continents and is the cause of important economic losses. An initial asymptomatic nonexcretory stage of the disease evolves to an asymptomatic excretory stage and finally to a symptomatic excretory stage, in which the clinical symptoms of chronic diarrhea with shedding of large quantities of bacteria are predominant (7).

Since infected cattle at an early stage of paratuberculosis outnumber those at a late stage, early diagnosis is crucial to identify potential bacterial shedders and to avoid the propagation of infection. The available diagnostic tests have serious limitations. In fact, microbiological identification is of limited use, because the replication of this mycobactin-dependent organism is slow (positive cultures are obtained after 2 to 4 months) (23). The use of specific nucleic acid probes allows identification of *M. paratuberculosis* in feces without preliminary culture of the microorganism but cannot pick out nonexcretory infected animals (31). On the other hand, cutaneous testing with extracts of either *M. paratuberculosis* (johnin) or *Mycobacterium avium* (avian tuberculin) lacks specificity and yields negative reactions with advanced cases (7, 16). Immunoenzymometric serological tests (ELISA) that monitor the levels of antimycobacterial antibodies in infected cattle seem to diagnose the disease correctly; however, serological tests with specific *M. paratuberculosis* antigens are not yet available (7, 26).

We have previously analyzed the immunological properties of A36, a major antigen complex of *M. paratuberculosis*. An A36-based ELISA was applied to serological analysis of paratuberculosis: it correctly diagnosed the infected cattle and yielded negative values with healthy animals (10). This test, however, lacked species specificity, because A36 cross-reacts with similar antigens present in other mycobacteria. A36 contains some 30 proteins (20 to 90 kDa), whose immunological activities in humoral (10) and cellular (13) immunity have been explored. Components in the 28- to 45-kDa range are the most antigenic for both cellular immunity and humoral immunity. The 34-kDa protein of the A36 complex was found to contain B-cell epitopes specific with respect to *M. avium*, *Mycobacterium bovis*, and *Mycobacterium phlei* and to be recognized by both hyperimmune rabbits and paratuberculous cattle (10, 13).

In the present work, a genomic bank of *M. paratuberculosis* in an expression vector was prepared. Clones expressing polypeptides related to the 34-kDa protein were selected for production of *M. paratuberculosis*-specific epitopes usable for the development of a specific serological test for Johne's disease.

MATERIALS AND METHODS

Bacteria. Strains of *Escherichia coli* used as cloning hosts were Y1090 Δ(*lacU169*) *proA*$^+$ Δ(*lon*) *araD139 strA supF* (*trp C22*::Tn*10*) (pMC9) (r_k^- m_k^+) and MC1061 *araD139* Δ(*ara leu*)*7697* Δ(*lacX74*) *galU galK hsr hsm*^{22} *strA mcrA mcrB*. The other microorganisms analyzed as controls are described in Table 1.

Antisera. Anti-*M. paratuberculosis* rabbit serum was from Dako (Copenhagen, Denmark; lot no. 014). Sera from *M. paratuberculosis*-infected cattle were provided by M. Desmecht (National Institute for Veterinary Research, Brussels, Belgium) and B. Limbourg (Center for Veterinary Medicine, Erpent, Belgium). Polyclonal antisera against *M. avium* serotype 4, *M. bovis* BCG, and *M. phlei* as well as those

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TABLE 1. Sources of analyzed microorganisms

Organism and strain	Origin[a]
Mycobacterium paratuberculosis	
2E	5
316F	5
ATCC 19698 (bovine)	6
ATCC 43015 (human)	6
2890 (bovine)	1
2891 (bovine)	1
2895 (goat)	1
17228/66 (bovine)	2
Mycobacterium avium	
D4	1
Serotype 4	1
Serotype 8	1
Serotype 2	1
Mycobacterium scrofulaceum	
Mycobacterium intracellulare	
M. avium-M. intracellulare-M. scrofulaceum complex	1
A3	4
A84	4
87537	4
Mycobacterium bovis BCG	10
Mycobacterium tuberculosis H37Rv	1
Mycobacterium phlei AM76	9
Mycobacterium leprae	8
Mycobacterium fortuitum M62	1
Mycobacterium smegmatis	1
Mycobacterium gordonae ATCC 14430	6
Corynebacterium xerosis	1
Nocardia asteroides	1
Brucella abortus B3	3
Salmonella typhimurium	3
Leprosy-derived corynebacteria LDC 15	7

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against the mycobacterial antigen complex A36 and the a362 polypeptide (a recombinant mycobacterial polypeptide fused to β-galactosidase and expressed by the λgt11-a362 clone) were produced by repeated subcutaneous inoculations into rabbits (10 μg of proteins per 0.5 ml of buffered saline emulsified with an equal volume of incomplete Freund's adjuvant; six inoculations at 1-week intervals).

Purification of *M. paratuberculosis* DNA. Suspensions of bacteria (10 mg [wet weight] in 0.5 ml of 100 mM NaCl–1 mM EDTA–50 mM Tris-HCl [pH 7.8]) were incubated sequentially with lysozyme (1 mg/ml; 3 h at 37°C and then 14 h at 50°C), pronase (1 mg/ml; 1 h at 37°C), and sodium dodecyl sulfate (SDS; 1% [wt/vol]; 1 h at 37°C). Mixtures were extracted with chloroform-isoamyl alcohol (24:1 [vol/vol]), water-saturated phenol, and ether. After incubation with RNase (10 μg; 1 h at 37°C), DNA was loaded on a column of Sephadex G50 (equilibrated with 4.8 mM sodium phosphate, pH 6.8) and purified on hydroxyapatite (washed with 8 M urea–0.1 M sodium phosphate buffer [pH 6.8] containing 1% SDS and then with 4.8 mM sodium phosphate [pH 6.8] and eluted with 480 mM sodium phosphate [pH 6.8]).

Construction of a λgt11 genomic library of *M. paratuberculosis*. The DNA of *M. paratuberculosis* ATCC 19698 (22) was sheared by sonication to segments with average lengths of 0.5 to 1.5 kb (Vibra Cell ultrasonicator; 60 W, 2 s). Shearing was monitored by agarose gel electrophoresis. *Eco*RI sites were methylated with *Eco*RI methylase (5 μg of sheared DNA in 50 μl of buffer containing 50 mM Tris-HCl [pH 7.5], 1 mM Na_3EDTA, 5 mM dithiothreitol, 50 μM *S*-adenosyl-L-methionine, and 200 U of *Eco*RI methylase per ml). Methylation was pursued for 30 min at 37°C and stopped by 10 min of incubation at 70°C. The methylation reaction mixture was adjusted to a final concentration of 0.01 M $MgCl_2$; 0.06 mM (each) dATP, dCTP, dGTP, and dTTP; and 0.02 M $(NH_4)_2SO_4$. DNA fragments in 40 μl of this mixture were blunt ended by incubation at 37°C for 20 min with 20 U of T4 DNA polymerase. Na_3EDTA (15 mM, final concentration) was added, the reaction mixture was extracted twice with phenol-chloroform, and the aqueous phase was extracted with ether. After addition of sodium acetate (0.3 M, final concentration), DNA was precipitated with 2 volumes of ethanol at −20°C and washed with 70% ethanol. The DNA pellet was dissolved in 10 μl of 20 mM $MgCl_2$–20 mM dithiothreitol–100 mM Tris-HCl (pH 7.5) buffer containing 200 μg of phosphorylated *Eco*RI linkers (GGAATTCC) per ml. After addition of polyethylene glycol 6000 (15% [vol/vol], final concentration), ATP (1 mM, final concentration), and 2 U of T4 DNA ligase, the reaction mixture was incubated overnight at 12°C. The action of the ligase was stopped by 10 min of incubation at 70°C, and the mixture was incubated at 37°C with 80 U of *Eco*RI. Mycobacterial DNA fragments purified from linker excess on Sephadex G25 were extracted sequentially with phenol-chloroform and ether, precipitated with ethanol, and dissolved in TE buffer (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA). Mycobacterial DNA (0.5 μg) was ligated (18 h at 12°C) with 1 μg of dephosphorylated *Eco*RI-digested λgt11 DNA (Promega) (32) in 5 μl of 10 mM $MgCl_2$–10 mM dithiothreitol–1 mM ATP–66 mM Tris-HCl (pH 7.5) buffer containing 1 U of T4 DNA ligase. Phage packaging of cloned DNA was obtained with the gigapack extract (Stratagene, La Jolla, Calif.).

Immunological screening of the λgt11 *M. paratuberculosis* genomic library. Overnight cultures of *E. coli* Y1090 in Luria-Bertani medium supplemented with 2% (wt/vol) maltose, 10 mM $MgSO_4$, and 100 μg of ampicillin per ml were pelleted, suspended in 10 mM $MgSO_4$ to an optical density of 1 at 600 nm, and then infected with recombinant bacteriophages (multiplicity of infection, 5 at 37°C for 20 min). Infected cells were then plated in soft agar (Luria-Bertani medium containing 0.8% [wt/vol] agar, 10 mM $MgSO_4$, and 100 μg of ampicillin per ml). The plates were incubated for 3 h at 42°C, overlaid with isopropyl-β-D-thiogalactopyranoside (IPTG)-saturated nitrocellulose, and incubated for 18 h at 37°C. Filters were removed, washed with TBS buffer (0.15 M NaCl, 0.010 M Tris-HCl [pH 7.5]), overlaid with the same buffer containing 3% (wt/vol) gelatin, and then incubated with the sera to be analyzed. These rabbit or bovine sera were preabsorbed with a lysate of *E. coli* Y1090 diluted with TBST buffer (TBS buffer containing 0.05% [vol/vol] Tween 20 and 1% [wt/vol] gelatin). After being washed, the filters were incubated for 1 h with 1/400 dilutions of peroxidase-labeled anti-rabbit or anti-cow immunoglobulin (Ig) (Dako). After repeated washings with TBST and TBS, the peroxidase substrate α-chloronaphthol (BioRad Laboratories, Richmond, Calif.) and hydrogen peroxide were added. The reaction was stopped by washing the filters with distilled water. Plaques corresponding to the reactive spots on the filters were transferred to SM medium (100 mM NaCl, 10 mM $MgSO_4$, 20 mM Tris-HCl [pH 7.4]) and purified by repeated passages on *E. coli* Y1090.

Affinity purification of antibodies directed against recombi-

nant mycobacterial proteins. After infection of *E. coli* by λgt11 recombinant phages, the plates were incubated for 3 h at 42°C, overlaid with IPTG-saturated nitrocellulose, and incubated for 18 h at 37°C. The filters were then removed and washed with TBS buffer (pH 7.5), incubated for 30 min with TBS buffer (pH 7.5) containing 3% (wt/vol) gelatin, and then incubated for 3 h with anti-A36 polyclonal Ig previously absorbed (18 h at 4°C) with a lysate of *E. coli* Y1090. After repeated washings with TBST, bound Ig was eluted (3.5 ml of 0.2 M glycine-HCl buffer [pH 2.5]; 2 min at 18°C), neutralized (1.75 ml of 1 M K_2HPO_4-KH_2PO_4 buffer [pH 9.0] containing 5% [vol/vol] fetal calf serum), and diluted 1:1 with distilled water. For storage, fetal calf serum (20% [vol/vol]) was added.

Preparation of lysates from recombinant bacteria. For enhanced expression, λgt11 inserts were subcloned into the *Eco*RI site of the expression vector $pUEX_2$ (3), which was used to transform *E. coli* MC1061. Single colonies of transformed *E. coli* were grown at 30°C to an A_{600} of 0.3 and then heat shocked (90 min at 42°C). Harvested cells were disrupted by sonication and frozen in liquid nitrogen.

SDS-PAGE. Proteins of the A36 complex and those produced by recombinant phages were analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (19). Plaque-purified recombinant phages were used to infect *E. coli* Y1090. The phage-cell mixtures were plated in soft agar (Luria-Bertani medium containing 0.8% agar, 100 μg of ampicillin per ml, and 10 mM IPTG). The plates were incubated for 4 h at 42°C and then for 1 night at 37°C. Approximately 10 plaques of each recombinant phage were removed and suspended in 100 μl of SDS-PAGE loading buffer (0.125 M Tris-HCl [pH 6.8], 5% [wt/vol] SDS, 20% [vol/vol] glycerol, 10% [vol/vol] β-mercaptoethanol, 0.05% [wt/vol] bromophenol blue) (25). A36 preparations (50 μg of proteins) and proteins from lysis plaques (10 to 20 μl) in gel loading buffer were boiled at 100°C for 5 min and loaded onto polyacrylamide gels.

Fractionation of phage proteins (on a 7.5% polyacrylamide gel) or A36 proteins (on a 12% polyacrylamide gel) was carried out at a constant current of 30 mA at 20°C in a vertical electrophoresis unit (gel was 16 by 18 cm; Hoefer Scientific Instruments, San Francisco, Calif.). Molecular weight protein markers (Sigma, St. Louis, Mo.) were myosin (205.0 kDa), β-galactosidase (116.0 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa), carbonic anhydrase (29.0 kDa), trypsinogen (24.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa). Protein bands were stained with Coomassie brilliant blue.

Immunoblot analysis. A36 electrophoresed components were transblotted (LKB 217 Multiphor 2; Bromma, Sweden; 100 mA; 2 h; with buffer containing 20% methanol, 0.039 M glycine, and 0.048 M Tris base [pH 8.8]) onto nitrocellulose membranes (BA 85; Macherey-Nagel, Dueren, Germany). A36 proteins were visualized by sequential incubation with recombinant polypeptide affinity-purified antibodies and peroxidase-labeled anti-rabbit Ig (Dako; 1/400 dilution). Photography was done under UV light (254 nm) (11). A similar technique was used for dot blot experiments; i.e., spots of sonicated microorganisms on nitrocellulose membranes were incubated with anti-a362 rabbit antiserum and peroxidase-labeled anti-rabbit Ig. Reference samples of transblotted total proteins were visualized by colloidal gold staining as described by the producer (Aurodye forte; Amersham, Slough, United Kingdom) (24).

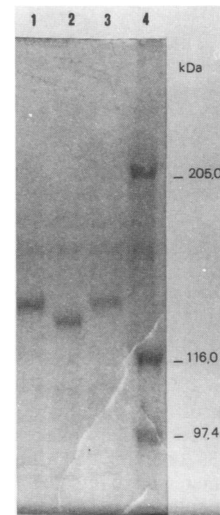

FIG. 1. Size evaluation of recombinant polypeptides of *M. paratuberculosis*. Polypeptides within the lysis plaques produced by the λgt11 recombinant phages a361 (lane 1), a362 (lane 2), and a363 (lane 3) were fractionated by acrylamide gel electrophoresis (SDS-7.5% PAGE) and stained with Coomassie blue. Molecular weight markers of the indicated sizes are in lane 4.

Serological analysis (ELISA) with recombinant polypeptides. Multiwell microtiter plates (Microwell Module; high binding capacity; Nunc, Roskilde, Denmark) were coated either with purified thermostable macromolecular antigen (TMA) complex (0.1 μg in 100 μl of 0.05 M Na carbonate buffer [pH 9.6] per well) or with lysates of *E. coli* MC1061 producing recombinant polypeptides (4 μg in 100 μl of 0.05 M Na carbonate buffer [pH 9.6] per well), air dried overnight, and saturated (400 μl of 0.1% [wt/vol] serum albumin-0.15 M NaCl solution per well; 1 h at 37°C). After incubation (100 μl per well; 1 h at 37°C) of bovine sera diluted 1/400 in PBST (0.15 M NaCl-0.02 M phosphate buffer [pH 7.2] containing 0.005% Tween 80), washing with PBST, and addition of peroxidase-labeled rabbit anti-cow Ig (Dako; 100 μl of 1/400 dilution in PBST per well; 1 h at 37°C), excess reagent was removed by PBST washings, and peroxidase substrate was added (100 μl of a 17 mM Na citrate buffer [pH 6.3] containing 0.2% [wt/vol] *O*-phenylenediamine and 0.015% [wt/vol] hydrogen peroxide per well; 30 min at 37°C in the dark). The reaction was stopped by the addition of 100 μl of 2 M H_2SO_4 per well, and the A_{492} was measured in a colorimetric plate reader (SLT 210; Kontron Analytical, Watford, United Kingdom).

Immune electron microscopy. Suspensions of mycobacteria in water (5×10^7 cells per 5 μl) were placed on carbon-Formvar 200-mesh copper grids and air dried. Grids were serially incubated with (i) bovine serum albumin (3% solution in buffered saline; 30 min at 37°C), (ii) rabbit anti-recombinant polypeptide a362 serum (a 10^{-3} dilution of Ig in buffered saline with 0.3% Tween 20; 2 h at 37°C), (iii) sheep anti-rabbit biotinylated Ig (1/200 dilution of Ig from Amersham in buffered saline-Tween 20; 1 h at 20°C), and (iv) gold-labeled streptavidin (a 1/20 dilution of a preparation from Amersham) (8). Grids were analyzed in a transmission electron microscope (Philips CM 10).

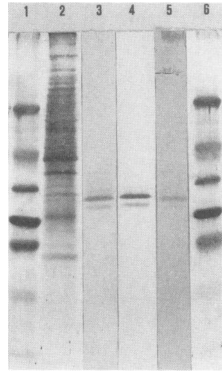

FIG. 2. Identification of A36 proteins corresponding to the recombinant polypeptides produced by phages a361, a362, and a363. The proteins of the A36 complex (50 μg) were fractionated by SDS–12% PAGE and transferred to nitrocellulose membranes which were either stained with colloidal gold (lane 2) or incubated with Ig purified from an anti-A36 serum by affinity to a single recombinant polypeptide present in phage lysis plaques (a361 in lane 3, a362 in lane 4, and a363 in lane 5). Bound primary Ig was revealed by a peroxidase-labeled second antibody. Molecular weight markers of 66.0, 45.0, 36.0, 29.0, 24.0, 20.1, and 14.2 kDa (lanes 1 and 6, top to bottom) were stained with colloidal gold. Photography was done under UV light (254 nm).

RESULTS

Isolation of recombinant λgt11 phages coding for portions of the 34-kDa protein of *M. paratuberculosis*. An *M. paratuberculosis* genomic bank in the expression vector λgt11 was prepared. Mycobacterial DNA was fragmented by sonication to segments of 500 to 1,000 bp, which, after the addition of *Eco*RI linkers, were cloned at the *Eco*RI site of the *lacZ* gene of λgt11. This genomic bank was immunoscreened with anti-A36 serum, and three recombinant phages (a361, a362, and a363) that produce polypeptides strongly reacting with this antiserum were selected.

To determine the sizes of the recombinant polypeptides, *E. coli* cells were infected with the recombinant phages, and synthesis of β-galactosidase fusion proteins was induced by IPTG. Proteins within the lysis plaques were fractionated by polyacrylamide gel electrophoresis (SDS–7.5% PAGE). Three β-galactosidase fusion proteins of 134.5 kDa (phage a361), 125.9 kDa (phage a362), and 133.0 kDa (phage a363) were identified (Fig. 1). After being transferred from the gel to nitrocellulose membranes, recombinant proteins were revealed with anti-A36 serum, suggesting the presence of at least one nonconformational epitope in each recombinant polypeptide (not shown).

The A36 proteins corresponding to each of the three recombinant polypeptides produced by phages a361, a362, and a363 were identified with the corresponding antibodies obtained as follows. Three *E. coli* cultures were infected (each with one of the recombinant phages) and plated. The plates were overlaid with IPTG-impregnated nitrocellulose membranes, and membrane-adhering recombinant proteins were incubated with anti-A36 serum (previously absorbed on a *E. coli* Y1090 lysate). The Ig directed against the recombinant proteins were eluted from the nitrocellulose membranes and incubated with A36 proteins that had been fractionated by electrophoresis on a 12% polyacrylamide gel (SDS-PAGE) and transblotted to a nitrocellulose membrane. The Western blot (immunoblot) displayed in Fig. 2 indicates that a couple of A36 proteins of 31 and 34 kDa were recognized by antibodies directed against the recombinant polypeptides produced by phages a361, a362, and a363 (the 31-kDa protein being the possible cleavage product of the 34-kDa one).

Analysis of antigenicities and specificities of recombinant polypeptides. The aim of this work was to produce segments of the 34-kDa protein to be used as specific diagnostic reagents. Accordingly, the recombinant polypeptides coded by the recombinant phages a361, a362, and a363 were tested against the sera of cattle affected by Johne's disease. As shown in Table 2, the recombinant polypeptides reacted with sera of animals bearing one of the clinical forms of the disease but not with sera from healthy bovines. The strongest reaction was afforded by clone a362.

Another requirement of paramount importance was specificity with respect to mycobacteria belonging to the saprophytic and pathogenic flora of cattle. Recombinant polypeptides were thus tested for reactivity with antisera against whole bacterial sonicates of *M. avium*, *M. bovis*, and *M. phlei*. Data in Table 2 indicate that none of the three polypeptides shares epitopes with *M. phlei*. Polypeptide a363 shares epitopes with *M. bovis* and *M. avium*, and polypeptide a361 shares epitopes with *M. avium* but not with *M. bovis*. The peptide produced by phage a362 contains epitopes found in *M. paratuberculosis* but not in *M. avium* or *M. bovis*.

In conclusion, only one of the selected clones (a362) produced a recombinant polypeptide which fulfilled both requirements of species specificity and relevance to Johne's disease. The remaining part of the present work was restricted to the characterization and use of the recombinant polypeptide produced by clone a362.

Localization of epitopes corresponding to the a362 polypeptide at the *M. paratuberculosis* surface. The presence of the A36 complex at the cell surface of *M. paratuberculosis* has been previously reported (10). An antiserum directed against

TABLE 2. Analysis of antigenicities and specificities of *M. paratuberculosis* polypeptides produced by recombinant λgt11 clones

Recombinant phage	Antigenicity[a] at the indicated stage of disease				Cross-reaction[b]		
	0	1	2	3	*M. avium*	*M. bovis*	*M. phlei*
a361	−	++	++	+++	+	−	−
a362	−	+++	+++	+++	−	−	−
a363	−	+	++	++	+	+	−

[a] The degree of antigenicity was evaluated from the immunoblot intensity with the corresponding sera: no (−), low (+), medium (++), or high (+++) antigenicity. Sera were from an uninfected bovine (stage 0) and from cattle at different stages of Johne's disease: asymptomatic nonexcretory (stage 1), asymptomatic excretory (stage 2), and symptomatic excretory (stage 3) forms.

[b] The presence (+) or absence (−) of a cross-reactive epitope(s) with respect to the indicated mycobacteria was tested with rabbit antisera directed against the corresponding bacterium.

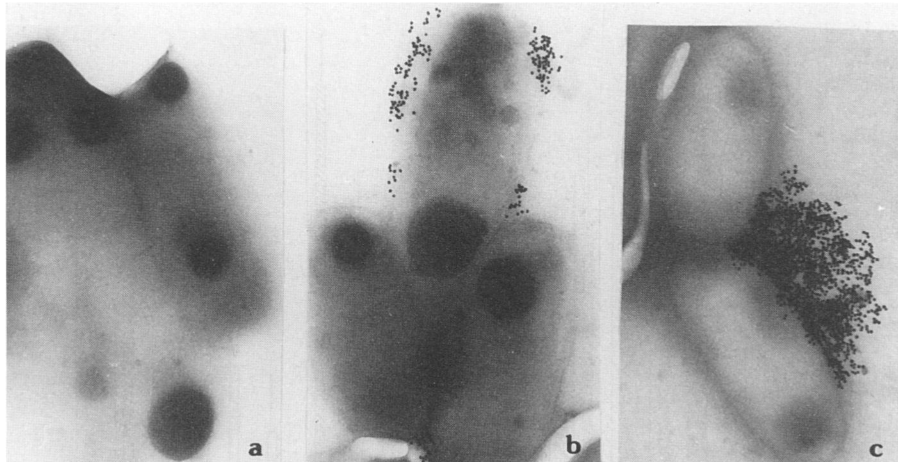

FIG. 3. Localization of epitopes corresponding to the a362 polypeptide at the *M. paratuberculosis* surface. Exponential-phase (a, b) and stationary-phase (c) *M. paratuberculosis* cells on microscope grids were incubated with sera from rabbits inoculated with a 362 recombinant polypeptide (b and c) or not inoculated (a). Samples were incubated with sheep anti-rabbit biotinylated Ig and gold-labeled streptavidin and photographed with a transmission electron microscope.

the recombinant polypeptide a362 was used to check the peripheral locations of epitopes belonging to the portion of the 34-kDa protein expressed by clone a362. For this purpose, the mycobacterial DNA cloned at the *Eco*RI site of recombinant phage a362 was cleaved and subcloned at the *Eco*RI site of expression vector $pUEX_2$. The resulting recombinant plasmid was used to transform the MC1061 strain of *E. coli* to produce large quantities of the recombinant a362 polypeptide. The latter, after purification by gel electrophoresis (SDS–7.5% PAGE), was injected into a rabbit for the preparation of anti-a362 serum. Electron micrographs show the presence of these epitopes at the cell surface (Fig. 3b) and their release in extracellular fluid during stationary growth phage (Fig. 3c).

Assessment of species specificity of the recombinant polypeptide a362. The recombinant polypeptide a362 was analyzed for specificity with respect to a series of *M. paratuberculosis* and *M. avium* strains. Some strains of corynebacteria, mycobacteria, and nocardia (the CMN group of microorganisms) and two bacteria pathogenic for bovines were also considered. The dot blot experiment illustrated in Fig. 4 was carried out by spotting lysates of different bacterial strains onto a nitrocellulose membrane. Membranes were then incubated successively with anti-a362 rabbit serum (see the section above) and, after washing, peroxidase-labeled swine anti-rabbit IgG. Spots were revealed by the peroxidase reaction. The control samples (spot 18 in Fig. 4) confirmed the cross-reactivity of a362 recombinant polypeptide with the A36 complex. All eight *M. paratuberculosis* isolates (spots 1 to 8) were positive, whereas the closely related organisms of the *M. avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* group (spots 9 to 17) were negative. No positive reaction was given by other tested bacteria (spots 19 to 26 and 28 to 30). Surprisingly, one strain of corynebacteria yielded a positive reaction (spot 27).

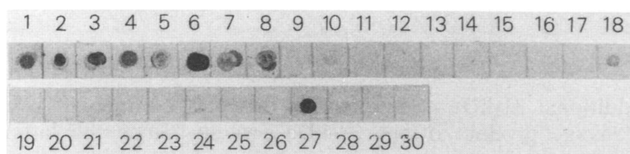

FIG. 4. Specificity of recombinant polypeptide a362 with respect to different microorganisms. Lysates of different bacteria (2 μg of protein per sample) and the antigen complex A36 from *M. paratuberculosis* (2 μg of protein per sample) were spotted onto a nitrocellulose membrane. After incubation with anti-a362 rabbit serum and washing, blots were stained with peroxidase-labeled anti-rabbit swine Ig. Samples: *M. paratuberculosis* 2E (lane 1), 316F (lane 2), ATCC 19698 (lane 3), ATCC 43015 (lane 4), 2890 (lane 5), 2891 (lane 6), 2895 (lane 7), and 17228/66 (lane 8); *M. avium* serotype 4 (lane 9), strain D4 (lane 10), serotype 2 (lane 11), and serotype 8 (lane 12); *M. intracellulare* (lane 13); *M. scrofulaceum* (lane 14); *M. avium-M. intracellulare-M. scrofulaceum* A3 (lane 15), A84 (lane 16), and 87537 (lane 17); *M. bovis* BCG (lane 19); *Mycobacterium tuberculosis* H37Rv (lane 20); *M. phlei* AM76 (lane 21); *Mycobacterium leprae* (lane 22); *Mycobacterium fortuitum* M62 (lane 23); *Mycobacterium gordonae* ATCC 14430 (lane 24); *Mycobacterium smegmatis* (lane 25); *Nocardia asteroides* (lane 26); *Corynebacterium xerosis* (lane 27); LDC 15 (lane 28); *Brucella abortus* B3 (lane 29); *Salmonella typhimurium* (lane 30). Sample 18 was the A36 complex of *M. paratuberculosis*.

Use of recombinant polypeptide a362 in ELISA for paratuberculosis. The sonic extract of *E. coli* MC1061, which was transformed by recombinant plasmid $pUEX_2$ expressing the a362 polypeptide, was used to coat microtiter plates. Control plates were coated with A36 and with sonicates of *E. coli* containing the nonrecombinant $pUEX_2$ plasmid. Series of sera from healthy cattle (7 samples) and ill cattle (25 bovines at different stages of the disease) were tested. These sera were preabsorbed on sonic extracts of strain MC1061 of *E. coli* transformed with the nonrecombinant $pUEX_2$ plasmid (to avoid the reaction of anti-*E. coli* Ig present in bovine sera with *E. coli* proteins present in the recombinant a362 polypeptide preparation). Data displayed in Fig. 5 indicate that all the sera of infected bovines (at all stages of the disease) yielded positive values not only with the A36 complex but also with the recombinant polypeptide a362.

DISCUSSION

Although several *M. paratuberculosis* antigens have been identified (1, 2, 4, 6, 9, 14, 17, 29, 30) only a few of them,

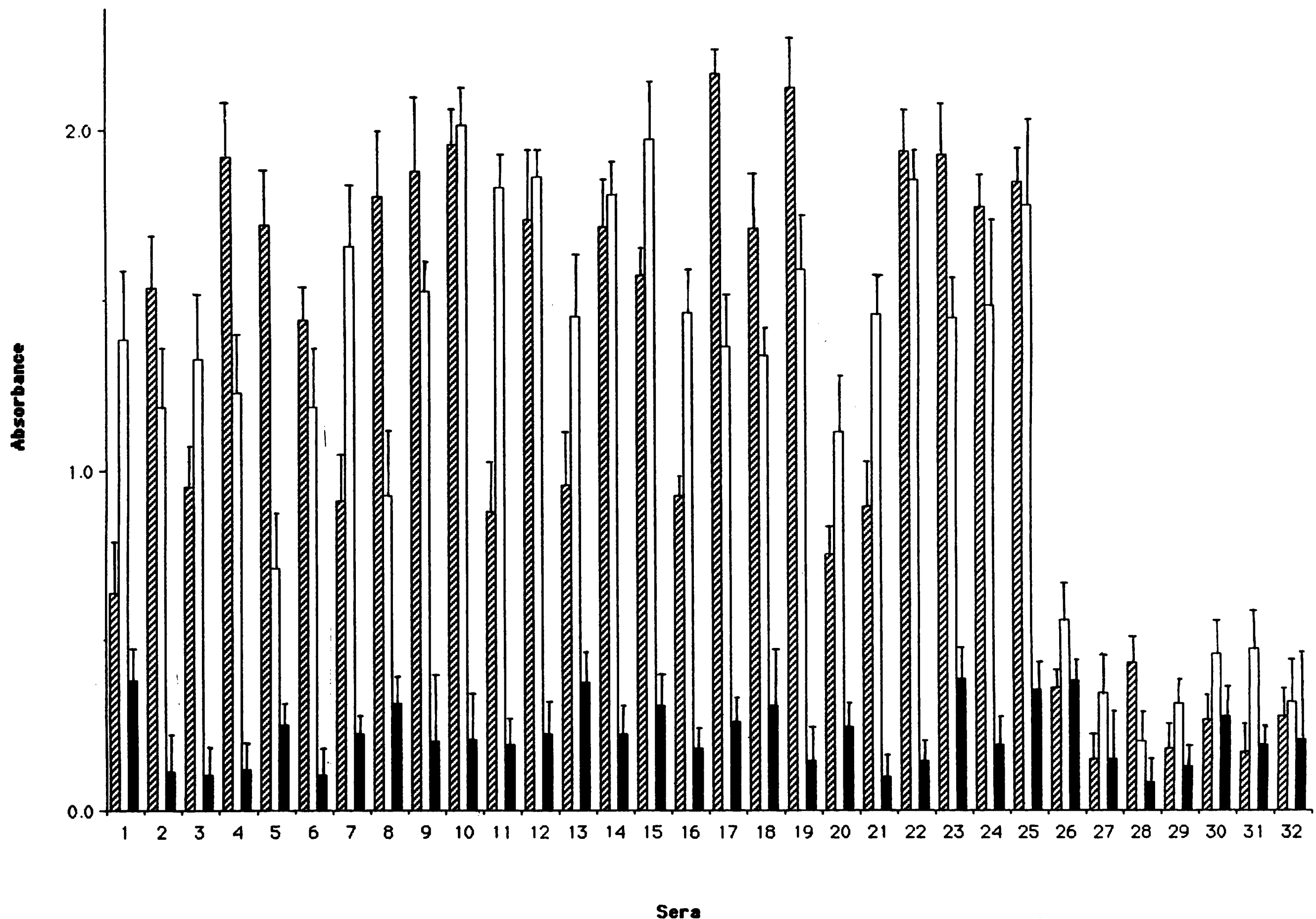

FIG. 5. Comparative serological analyses of paratuberculous bovines with the a362 recombinant polypeptide and the A36 complex. A comparative survey of healthy and infected animals used, respectively, the A36 complex (0.1 μg per well; ▧) or the recombinant a362 polypeptide (4 μg of a sonic extract of pUEX$_2$-a362-transformed *E. coli* MC1061 per well; □) as reagents for an ELISA. A sonic extract of nonrecombinant pUEX$_2$-transformed *E. coli* MC1061 was used as a blank (■). Sera (1/400 dilutions) from infected animals at stage I (asymptomatic nonexcretory) (sample 1), stage II (asymptomatic excretory) (samples 2 to 13), and stage III (symptomatic excretory) (samples 14 to 25) and from healthy cattle (samples 26 to 32) were analyzed. The mean absorbances (A_{492}) of three experiments with standard deviations are reported. Cutoff values were calculated as means plus three times the standard deviations: 0.580 for A36 ELISA and 0.747 for a362 ELISA.

including antigens A (27), D (5, 27), A36 (10), and LAM (28), were purified and used in ELISAs. However none of them was specific with respect to *M. paratuberculosis*. A36 produces a single precipitinogen arc in crossed immunoelectrophoresis (10, 14), but SDS-PAGE fractionation of this antigen yields some 30 components of 20 to 90 kDa, one-third of which are recognized by the sera of infected animals (10). One of the immunodominant proteins, the 34-kDa protein, contains epitopes specific with respect to all mycobacteria, including *M. avium*, the organism genetically more closely related to *M. paratuberculosis* (15, 18). Since the large-scale purification of this 34-kDa protein from bacterial homogenates is not practical, we used genetic engineering to obtain polypeptides to be used for serological tests.

The fact that the three recombinant polypeptides produced by the most antigenic clones present in our *M. paratuberculosis* bank corresponded to the 34-kDa protein suggests the immunodominance of this protein (Fig. 2). Antibodies directed against these three recombinant polypeptides also recognized a 31-kDa component (Fig. 2). The simplest explanation for the immunostaining of such an additional 31-kDa component is that this component is a cleavage product of the 34-kDa protein. Since the latter apparently is presented at the cell surface and released extracellularly (Fig. 3), the cleavage of a hypothetical signal peptide is conceivable.

The recombinant polypeptides produced by the selected clones a361, a362, and a363 are likely to correspond to different regions of the 34-kDa protein. The different specificity levels of the recombinant polypeptides produced by the three clones with respect to *M. paratuberculosis*, *M. avium*, and *M. bovis* (Table 2) suggest the occurrence in the 34-kDa protein of at least three epitopes. One of them, fully specific for *M. paratuberculosis*, is located in the polypeptide coded for by the mycobacterial gene portion cloned in phage a362 (a segment 500 bp long; data not shown). Another epitope, shared by *M. paratuberculosis* and *M. avium*, is coded for by the mycobacterial gene portion cloned in phage a361 (1,000 bp; data not shown), whereas the epitope cross-reacting with *M. paratuberculosis* and *M. bovis* is coded for by the mycobacterial DNA segment inserted in phage a363 (500 bp; data not shown). The

presence in the 34-kDa protein of a specific epitope was previously demonstrated (10), while the occurrence of additional cross-reacting epitopes is shown in this work: proteins homologous to the 34-kDa component are thus expected to be present in both *M. bovis* and *M. avium*. Moreover, since the DNA insert in phage a362 hybridized with *M. avium* DNA but not with *M. bovis* DNA (not shown), the *M. paratuberculosis* 34-kDa protein seems to be more closely related to the homologous protein of *M. avium* than to that of *M. bovis*.

The immunodominance of the recombinant polypeptide produced by phage a362 (Fig. 5) and its species specificity (Table 2 and Fig. 4) support its use as a reagent for a serological test for Johne's disease. A cross-reaction with *Corynebacterium xerosis* (spot 27 in Fig. 4) is observed; although this organism is not pathogenic for bovines, other corynebacteria (*Corynebacterium renale* and *Corynebacterium equi*, for instance) may cause pyelonephritis and lymphadenitis in cattle (12, 20, 21). Consequently, a larger screening of microorganisms of the CMN group (genera *Corynebacterium*, *Mycobacterium*, and *Nocardia*) needs to be performed to identify possible cross-reacting antigens. An A36-based ELISA was previously shown to diagnose all the tested forms of paratuberculosis correctly (10). The preliminary assays whose results are displayed in Fig. 5 attest to an equally good performance of the a362-based ELISA. Since the number of cases included in these preliminary trials is insufficient to assess the possibilities of this test, a wider screening of both uninfected and infected cattle is presently in progress.

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