Comparative Antigenic Analysis of "Gordona aurantiaca" and Mycobacterium fallax

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Treatment of mycobacterial cells with Triton X-100 allowed the extraction and solubilization of antigens. Such extracts provided species-specific crossed immunoelectrophoresis profiles which demonstrated that "Gordona aurantiaca" and Mycobacterium fallax are antigenically distinct. Using the Mycobacterium bovis BCG antiserum as a reference, we showed that M. fallax and M. bovis BCG are more related to each other than "G. aurantiaca" is to both species.

In 1971, Tsukamura created the genus "Gordona" for slightly acid-fast organisms isolated from the sputa of patients and from soil (20). The species "Gordona bronchialis", "G. rubra", "G. terraæ", and "G. aurantiaca" were described (20, 23). Subsequently, a numerical taxonomy study showed that this genus also contains the species Rhodococcus rhodochrous, and it was proposed to change the generic name "Gordona" to Rhodococcus (9). The species "G. aurantiaca" was named Rhodococcus aurantiacus and was recently formally revived (25). However, from the first description in 1971 (23), "G. aurantiaca" has had an equivocal taxonomic position. Phenetic studies showed that "G. aurantiaca" forms a homogeneous group distinct from the aggregate clusters equated with the genera "Mycobacterium", Nocardia, and Rhodococcus (11, 21, 22, 24). Several features, such as rather strong acid fastness, inability to reduce nitrate, β-galactosidase activity, and resistance to 5-fluorouracil and mitomycin C, characterize "G. aurantiaca" as an irregular taxon in the genus "Gordona" (25). In addition, chemical and serological analyses showed that "G. aurantiaca" presents specific mycolic acids (11, 19), completely unsaturated menaquinones (11), and has a characteristic distribution of precipitinogens (17). According to these findings, it was suggested that "G. aurantiaca" might belong to another genus (11, 17).

The mycolic acids of "G. aurantiaca" are of special interest, because the analysis of mycolic acid composition has been widely used as a source of valuable information for the determination of the genera Corynebacterium, Mycobacterium, Nocardia, and Rhodococcus (10). A single mycobacterial species, Mycobacterium fallax (13), and "G. aurantiaca" both contain high-molecular-weight polysaturated mycolates (11, 13, 16, 19), which can be considered as intermediate kinds between the well-defined mycolates characteristic of the above-mentioned genera. Consequently, "G. aurantiaca" and M. fallax appeared to be borderline species in at least three genera (Mycobacterium, Nocardia, and Rhodococcus). Therefore, we thought it would be of interest to examine the antigenic relationships of "G. aurantiaca" and M. fallax.

Another purpose of this paper was to test the isolation of bacterial, specifically mycobacterial, antigens by differential extraction with a detergent such as Triton X-100. Historically, filtrates of cultures were the first sources of mycobacterial antigens, but today most of the immunologic studies applied to mycobacterial taxonomy require cell disruption and use whole-cell extracts. The analysis of such complex mixtures is difficult, because it is hampered by numerous antigens shared by all species. To isolate individual antigens, many purification techniques have been used, based on antigen solubility, molecular charge, molecular size, affinity for different substrates, or, recently, by using monoclonal antibodies (7, 8). Only a few investigators tried to purify a limited set of antigens by selective extraction. Phenol-soluble antigens were extracted from M. gastri, M. kansasi, and M. marinum, and antigens characteristic of each species were determined (26). Antigenic surface proteins from M. smegmatis were solubilized with Triton X-100 (3). As this latter method was easy to handle, we evaluated it as a taxonomic tool.

MATERIALS AND METHODS

Strains. The mycobacterial strains were from our own collection (Table 1), "G. aurantiaca" M292 was kindly supplied by M. Goodfellow (Department of Microbiology, University of Newcastle upon Tyne, United Kingdom), who received the strain initially as "G. aurantiaca" 4409 from M. Tsukamura (National Chubu Hospital, Obu, Aichi, Japan). The cells were grown in Trypto-Casein-Soya broth (Institut Pasteur Production, Marnes la Coquette, France) for 1 week, except for the M. tuberculosis and M. bovis BCG strains which were grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, Mich.) for 4 weeks. The strains were incubated at 37°C except for the M. fallax and M. chelonae strains which were incubated at their optimal temperature of 30°C.

Antigens. Bacteria were harvested by centrifugation and were washed several times in phosphate-buffered saline (pH 7.2). Cells (about 1 g [wet weight]) were then suspended in 1 ml of phosphate-buffered saline containing 20 mM ethylenediaminetetraacetic acid and 0.5% (wt/vol) Triton X-100 (Prolabo, Paris, France). The antigens were extracted by shaking for 2 h at room temperature. The supernatant fluids were collected after centrifugation for 1 h at 20,000 × g. In addition to the Triton X-100 extract, a sonic extract was prepared from M. bovis BCG cells. This extract was obtained by sonicating the cell suspension (1 g [wet weight] in 1 ml of phosphate-buffered saline) for 30 min (15 × 2 min) with a 20/200 SV sonicator (Biorad Scientific, Strasbourg, France). The extract was then diluted to 1 mg of protein per ml. The protein concentrations were determined by the method of Lowry et al. (14) with bovine serum albumin as a standard.

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Antisera. The different antigenic extracts were adjusted to 1 mg of soluble proteins per ml and mixed with an equal volume of Freund incomplete adjuvant. The mixtures (2 ml) were injected intradermally in rabbits at multiple sites. After 3 weeks, the immunization was continued by subcutaneous injections of the same dose every 2 weeks. The rabbits were bleed when adequate titers of serum antibodies were obtained by an immunodiffusion technique (15). Four antisera were prepared against diluted bacterial antigens; a sonic extract from M. bovis BCG and Triton X-100 extracts from M. bovis BCG, "G. aurantiaca" M292, and M. fallax CIP 8139T.

SDS-PAGE. The patterns of the proteins were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (12). The low-molecular-weight protein kit from Pharmacia, Uppsala, Sweden, was used as a standard.

CIE. Crossed immunoelectrophoresis (CIE) was performed by previously described methods (1, 2). The cathodic wells were filled with 20 μl each of antigenic extracts in the first-dimension electrophoresis. The antigens thus separated migrated into gels containing 20% (vol/vol) antiserum in the second-dimension electrophoresis; an intermediate and a top gel containing different antisera were also used (1).

Staining of plates. The plates were washed, dried, and stained with Coomassie blue.

RESULTS

Triton X-100 extraction of proteins. The concentrations of the proteins extracted by Triton X-100 are shown in Table 1. The results indicate that satisfactory yields of protein, between 0.78 and 1.42 mg/ml, could be obtained with an incubation of 2 h with 0.5% (wt/vol) Triton X-100. SDS-PAGE of the extracts showed more than 20 distinct bands, most of them corresponding to proteins having molecular weights within the range of 94,000 to 14,400 (Fig. 1). The protein profile was different for each test strain (Fig. 1A, B, and C).

CIE. The CIE patterns of M. fallax CIP 8139T and "G. aurantiaca" M292 in homologous systems indicate that the organisms are antigenically distinct (Fig. 2C and D). The antigenic mixtures of M. bovis BCG extracted by Triton X-100 or obtained after sonication were compared by CIE with the antisera prepared against the M. bovis BCG sonic extract (Fig. 2A and B). The homologous system was used as a standard reference; the pattern obtained is similar to the M. bovis BCG CIE profile previously published (5, 6). The number of precipitates was significantly lower with the Triton X-100 extract than with the antigenic mixture obtained after sonication of the cells, indicating that Triton X-100 did not extract all the cellular antigens.

Comparison of "G. aurantiaca" and M. fallax antigenic extracts. As shown previously (6, 18), cross-reactions are

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Protein (mg/ml)</th>
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<tbody>
<tr>
<td>M. tuberculosis CIP 140010001 (H37Rv, ATCC 27294T)</td>
<td>0.78</td>
</tr>
<tr>
<td>M. aurum CIP 141210001 (ATCC 23367)</td>
<td>0.84</td>
</tr>
<tr>
<td>M. smegmatis CIP 141330010 (ATCC 19420T)</td>
<td>0.92</td>
</tr>
<tr>
<td>M. bovis BCG CIP 140040001 (Pasteur strain)</td>
<td>0.96</td>
</tr>
<tr>
<td>M. chelonae CIP 140420003 (NCTC 358T)</td>
<td>1.04</td>
</tr>
<tr>
<td>&quot;G. aurantiaca&quot; M292</td>
<td>1.23</td>
</tr>
<tr>
<td>M. fallax CIP 141390005 (CIP 8139T, ATCC 35219)</td>
<td>1.42</td>
</tr>
</tbody>
</table>

* CIP, Collection Institut Pasteur Tuberculose, Paris, France; ATCC, American Type Culture Collection, Rockville, Maryland, United States; NCTC, National Collection of Type Cultures, London, United Kingdom; CIP, Collection Institut Pasteur, Paris, France.
FIG. 3. CIE patterns of "G. aurantiaca" M292 and M. fallax CIP 8139T antigens and various antisera. The antigens in the first-dimension electrophoresis were "G. aurantiaca" M292 (A) and M. fallax CIP 8139T (B) Triton X-100 extracts. The homologous antisera were incorporated in the top gels. Antisera incorporated in the intermediate gels were as follows: A, anti-M. fallax Triton X-100 extract; B and E, anti-M. bovis BCG sonic extract; C and F, anti-M. bovis BCG Triton X-100 extract; and D, anti-"G. aurantiaca" Triton X-100 extract.

detected by incorporating antibodies against other species in the intermediate gel of the CIE. The antigens extracted from one species but shared by another species are retained in the intermediate gel containing the serum against the heterologous species. The unshared antigens are detected in a top gel containing the antiserum against the homologous species. Fig. 3 shows how various antibodies reacted with the antigenic components of "G. aurantiaca" M292 (Fig. 3A to C) and M. fallax CIP 8139T (Fig. 3D to F). Only a few precipitates were identified with the heterologous systems containing either the antigenic extract from "G. aurantiaca" and the antiserum against M. fallax (Fig. 3A) or the antigenic extract from M. fallax and the antiserum against "G. aurantiaca" (Fig. 3D). The reference antiserum against the sonic extract of M. bovis BCG allowed the detection of more than 10 precipitates with the M. fallax antigenic extract (Fig. 3E) but produced only one slightly stained precipitate with the "G. aurantiaca" antigenic mixture (Fig. 3B). Such qualitative and quantitative differences were also apparent in the patterns obtained with the antiserum against the M. bovis BCG Triton X-100 extract (Fig. 3C and F). These data indicate a closer relationship between M. fallax CIP 8139T and M. bovis BCG than between "G. aurantiaca" M292 and either M. fallax CIP 8139T or M. bovis BCG. These antigens were suitable for antiserum production in rabbits. Because Triton X-100 treatment did not cause mycobacterial cell lysis, antigens detected in the extracts were probably surface antigens. However, further studies are necessary to establish the precise location of these antigens in the cell structure. We emphasize the usefulness of Triton X-100 extracts producing CIE patterns with no more than 20 precipitates which, as shown in this report, are sufficient to yield readily recognizable species-specific profiles.

CIE with intermediate and top gels containing different antisera was particularly well suited for the comparison of "G. aurantiaca" and M. fallax antigens. This method showed that few, if any, cross-reacting antigens were recognized, indicating that the two species are unrelated and clearly different. The comparison of "G. aurantiaca" and M. fallax antigens with antiserum prepared against the antigenic extracts of M. bovis BCG indicated that M. fallax is more related to M. bovis BCG than to "G. aurantiaca" (Fig. 3B and E). This is in agreement with the inclusion of the fallax species in the genus Mycobacterium (13) regardless of the uncommon mycolic acids synthesized by the strains of this species.

LITERATURE CITED

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