Identification of *Acinetobacter* Isolates from Species Belonging to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* Complex with Monoclonal Antibodies Specific for O Antigens of Their Lipopolysaccharides

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The unambiguous identification of *Acinetobacter* strains, particularly those belonging to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex, is often hindered by their close genomic and phenotypic relationships. In this study, monoclonal antibodies (MAbs) against the O antigens of the lipopolysaccharides from strains belonging to the *A. calcoaceticus-A. baumannii* complex were generated after the immunization of mice with heat-killed bacteria and shown by enzyme immunoassays and Western blotting to be specific for their homologous antigens. Since the *A. calcoaceticus-A. baumannii* complex comprises the most clinically relevant species, the MAbs were subsequently tested in dot and Western blots with proteinase K-treated lysates from a large collection of *Acinetobacter* isolates (*n* = 631) to determine whether the antibodies could be used for the reliable identification of strains from this complex. Reactivity was observed with 273 of the 504 isolates (54%) from the *A. calcoaceticus-A. baumannii* complex which were included in this study. Isolates which reacted positively did so with only one antibody; no reactivity was observed with isolates not belonging to the *A. calcoaceticus-A. baumannii* complex (*n* = 127). To identify additional putative O serotypes, isolates from the *A. calcoaceticus-A. baumannii* complex which showed no MAb reactivity were subjected to a method that enables the detection of lipid A moieties in lipopolysaccharides with a specific MAb on Western blots following acidic treatment of the membrane. By this method, additional serotypes were indeed identified, thus indicating which strains to select for future immunizations. This study contributes to the completion of a serotype-based identification scheme for *Acinetobacter* species, in particular, those which are presently of the most clinical importance.

*Acinetobacter* spp. have gained increased recognition in recent years as pathogens which have the potential to cause severe nosocomial infections in critically ill patients (1, 33). However, the quick and reliable identification of *Acinetobacter* strains has been hampered for a number of years due to the close phenotypic and genotypic relatedness of some species within the genus, particularly those of clinical relevance (1, 8). Based on DNA homology studies, 23 DNA groups (genomic species) are currently delineated, nine of which have received formal species names (3, 4, 12, 20, 32). Strains from genomic species 2 (*Acinetobacter baumannii*), 3, and 13 sensu Tjernberg and Ursing (13TU) (32) are frequently isolated from clinical specimens and are often associated with nosocomial outbreaks (1, 33); they belong, together with genomic species 1 (*Acinetobacter calcoaceticus*), to the so-called *A. calcoaceticus-A. baumannii* complex (11, 13). *A. calcoaceticus* strains are seldom isolated from patients or associated with infections (1). Other *Acinetobacter* strains are also isolated infrequently from patients, although both *Acinetobacter johnsonii* and *Acinetobacter baumannii* have been reported to be involved in cases of septicemia (2, 29). Due to the increased importance of *Acinetobacter* in the clinical environment and the lack of a reliable phenotypic method for the unambiguous identification of all members of the genus to the species level, we have focused on the development of an O-serotype-based identification scheme for *Acinetobacter*. Ongoing studies (22–27) have been centered mainly on the generation of O-antigen-specific monoclonal antibodies (MAbs) against strains belonging to the *A. calcoaceticus-A. baumannii* complex, but to date, these antibodies have been tested only on small numbers of clinical isolates within a limited geographical range.

Here, we report on the serological characterization of five MAbs which were generated against the O antigens of the lipopolysaccharides (LPSs) from strains belonging to species within the *A. calcoaceticus-A. baumannii* complex. These antibodies were tested together with three previously described O-antigen-specific MAbs (24, 25) on a large collection of strains which had been isolated from various clinical and environmental sources in different countries worldwide. The aim of the present study was to determine the extent to which the current panel of O-antigen-specific MAbs can be used for the identification of strains from the *A. calcoaceticus-A. baumannii* complex with monoclonal antibodies specific for O antigens of their lipopolysaccharides.
complex, the results of which will also be useful for the generation of future MAbs.

MATERIALS AND METHODS

Strains. A total of 504 Acinetobacter isolates belonging to the A. calcoaceticus-A. baumannii complex were investigated in the present study (Table 1). Additional Acinetobacter strains (n = 127) belonging to genomic species outside the A. calcoaceticus-A. baumannii complex were also examined (Table 2). Most strains had been characterized previously to the species level by DNA-DNA hybridization and/or by other methods such as amplified ribosomal DNA restriction analysis, ribotyping, and biotyping (4-7, 9, 12, 13, 16, 19, 30, 32, 34) and were obtained from various culture collections worldwide. The strains were originally isolated from different clinical and environmental specimens, e.g., blood, cerebrospinal fluid, sputum, urine, and soil. They were preserved in nutrient broth supplemented with 20% (vol/vol) glycerol at −80°C.

Bacterial LPS, whole-cell lysates, and proteinase K digestion. LPS was extracted by the phenol-water method (36) from the Acinetobacter strains against which MAbs were prepared (see below) and bohylphosed. Preparation of whole-cell lysates and proteinase K digestion were performed as reported elsewhere (22).

MAbs. The generation of MAbs was performed by immunizing BALB/c mice with heat-killed (100°C, 1 h) bacteria. Acinetobacter strains 7 (A. calcoaceticus) and 44 (genomic species 3), against which rabbit antisera had been produced in a previous study (22), were selected as immunogens. Acinetobacter strains ATCC 23055 (A. calcoaceticus) and ATCC 15308 (A. baumannii), purchased from the American Type Culture Collection (Manassas, Va.), as well as Acinetobacter strain NCTC 10303 (A. baumannii), purchased from the National Collection of Type Cultures (Central Public Health Laboratory, London, United Kingdom), were used as additional immunogens. These strains were selected additionally because they belong to the A. calcoaceticus-A. baumannii complex, thus making them interesting immunogens for this study, and are often used as reference strains for their respective species (1, 33). All MAbs were generated according to an immunization protocol described previously (24, 25) except that booster injections with the additionally selected immunogens were administered intravenously (via the tail vein). Only those animals whose serum exhibited the strongest reactivity with the respective immunogen in a dot blot assay were given booster injections, and their spleen cells were subsequently used for the generation of hybridoma cells. Hybridomas (one per antigen) which exhibited the highest EIA titers (expressed as the reciprocal value of the highest dilution of cell culture supernatant which yielded an optical density at 405 nm (OD405) of >0.2) were selected for further studies. They were cloned by limiting dilution (three times), isotypes, and purified by affinity chromatography on protein G. Purity was ascertained by Coomassie staining of SDS-PAGE gels (data not shown). MAb S51-3, against A. calcoaceticus strain 7, was of the immunoglobulin G1 isotype. MAbs S48-26, S53-1, S53-13, and S53-32, against Acinetobacter genomic species 3 strain 44, A. calcoaceticus strain ATCC 23055, A. baumannii strain ATCC 15308, and A. baumannii strain NCTC 10303, respectively, were of the immunoglobulin G3 isotype. The MAbs used in this study are bound LPS in 1% acetic acid and the detection of its lipid A moieties with MAb A6 (18) were carried out as described elsewhere (21).

RESULTS

Immunization of mice and preparation of MAbs. All BALB/c mice were immunized successfully. However, only those animals whose sera exhibited the strongest reactivity with the homologous antigen by dot blotting were given booster injections, and their spleen cells were subsequently used for the generation of hybridoma cells. Hybridomas (one per antigen) which exhibited the highest EIA titers (expressed as the reciprocal value of the highest dilution of cell culture supernatant which yielded an optical density at 405 nm (OD405) of >0.2) were selected for further studies. They were cloned by limiting dilution (three times), isotypes, and purified by affinity chromatography on protein G. Purity was ascertained by Coomassie staining of SDS-PAGE gels (data not shown). MAb S51-3, against A. calcoaceticus strain 7, was of the immunoglobulin G1 isotype. MAbs S48-26, S53-1, S53-13, and S53-32, against Acinetobacter genomic species 3 strain 44, A. calcoaceticus strain ATCC 23055, A. baumannii strain ATCC 15308, and A. baumannii strain NCTC 10303, respectively, were of the immunoglobulin G3 isotype. The MAbs used in this study are

| Genomic species | No. of isolates tested | No. of isolates reactive with the following MAb generated against the indicated species | No. of patterns obtained after acid hydrolysis | No. of isolates which did not react with any of the O-antigen-specific MAbs or with MAb A6 |
|----------------|-----------------------|------------------------------------------|---------------------------------------------|-----------------------------------------------|
| S51-3          | 11                    | 1                                       | 1                                           | 2 (18)                                       |
| S53-3          | 3                     | 3                                       | 3                                           | 5 (17)                                       |
| S48-3-13       | 2                     | 2                                       | 2                                           | 12 (11)                                      |
| S48-3-17       | 2                     | 2                                       | 2                                           | 12 (11)                                      |
| S53-13         | 2                     | 2                                       | 2                                           | 12 (11)                                      |
| S53-32         | 1                     | 1                                       | 1                                           | 13 TU                                        |
| S48-26         | 3                     | 3                                       | 3                                           | 33 (57)                                      |
| S48-13         | 1                     | 1                                       | 1                                           | 1 (5)                                        |

Values in parentheses are percentages of isolates. Acid-treated membrane-bound LPSs of proteinase K-digested bacterial lysates from Acinetobacter isolates which did not react with any of the O-antigen-specific MAbs used in this study were treated with MAb A6 (18).

For some isolates, we obtained a banding pattern different from the banding pattern obtained with the homologous strain (Fig. 2).

| Genomic species | Genus | Strain | Location | Species | Species Group |
|----------------|-------|--------|----------|---------|---------------|
| 4              | A. haemolyticus | 8 |
| 5              | A. junii | 20 |
| 6              | A. johnsonii | 14 |
| 7              | A. lwofii | 42 |
| 8/9            | A. radioresistens | 10 |
| 10             | 14BJ | 2 |
| 11             | 14TU | 7 |
| 12             | 15BJ | 1 |
| 13             | 15TU | 2 |
| 14             | 16 |
| 15             | 17 |

Values in parentheses are percentages of isolates. Acid-treated membrane-bound LPSs of proteinase K-digested bacterial lysates from Acinetobacter isolates which did not react with any of the O-antigen-specific MAbs used in this study were treated with MAb A6 (18).

Genomic species 8 and 9 are considered a single entity (32).
MAb in a Western blot. Lane 1, strain 24; lane 2, strain 34; lane 3, Acinetobacter genomic species 3; lane 4, Acinetobacter genomic species 3 strain 44; lane 5, A. calcoaceticus strain 7; lane 6, A. calcoaceticus strain ATCC 23055; lane 7, A. baumannii strain ATCC 15308; lane 8, A. baumannii strain NCTC 10303.

The results described below were obtained with affinity-purified MABs.

Specificities of MABs in EIAs. All MABs were highly specific for their homologous antigens, as determined by their reactivities in EIAs with purified LPS (5 μg/ml; 50 μl/well) as a solid-phase antigen. Antibody concentrations which yielded an OD₄₀₅ of >0.2 were between 0.5 ng/ml (MAb S51-3) and 630 ng/ml (MAb S53-13) with the homologous LPS (data not shown). None of the MABs reacted with any of the heterologous LPS preparations; no OD₄₀₅ of >0.2 was observed at antibody concentrations of up to 5 μg/ml for MABs S48-26, S51-3, S53-1, and S53-32 or up to 50 μg/ml for MAB S53-13. Checkerboard titrations showed that all antibodies bound to the homologous LPS over a wide range of antigen concentrations (0.01 to 10 μg/ml) (data not shown). The characterization of MABs S48-3-13, S48-3-17, and S48-13 have been reported previously (12, 13). The specificities of all antibodies were verified by Western blotting using a 10% separating gel (Fig. 1). A banding pattern characteristic of that of an O-polysaccharide chain was observed for all isolates when these were immunostained with the homologous antibody; none of the patterns were identical. No heterologous O-antigen reactivity was observed, and no reactivity with the core-lipid A moiety was observed when LPSs were separated on a 15% gel (data not shown), thus indicating that the generated MABs are specific for their homologous antigens.

The antibodies were subsequently tested in dot blots with proteinase K-treated bacterial lysates from all Acinetobacter isolates included in this study. Reactivity was observed with 273 of the 504 isolates from the A. calcoaceticus-A. baumannii complex used in this study (Table 1). None of the MABs reacted with any of the isolates from species outside of the A. calcoaceticus-A. baumannii complex (Table 2), and none of the isolates from the A. calcoaceticus-A. baumannii complex reacted with more than one antibody. Interestingly, MABs S51-3 and S53-32 also reacted with isolates not belonging to the same genomic species as that of the strain which was used for immunization (i.e., A. calcoaceticus and A. baumannii with MABs S51-3 and S53-32, respectively). The reactivities observed in dot blots could be confirmed by performing Western blot analysis. Although in most cases the obtained ladder patterns were indistinguishable from the pattern obtained with the respective homologous LPS (data not shown), a different banding pattern was observed for a number of isolates when they were immunostained with MAB S48-13 or MAB S51-3 (Fig. 2). Comparison of the core-lipid A moieties of these isolates with that of the homologous strain in silver-stained SDS–15% polyacrylamide gels showed no difference in migration distance (data not shown), thus suggesting that the O antigens of these isolates are antigenically related but structurally distinct.

Determination of O serotypes by acid hydrolysis. To identify additional putative O serotypes among those isolates from the A. calcoaceticus-A. baumannii complex which had not reacted with any of the MABs, a method was used to visualize any LPS via its lipid A moiety with a specific antibody (MAb A6) on Western blots following acidic treatment of the membrane-bound antigen. Twenty-eight O banding patterns were obtained (Table 1; Fig. 3): 2 were obtained for A. calcoaceticus; 13 were obtained for A. baumannii; 12 were obtained for Acinetobacter genomic species 3, and 1 was obtained for Acinetobacter genomic species 13TU. For some strains, no banding pattern was observed. The obtained patterns were different from the patterns that were observed for isolates from the A. calcoaceticus-A. baumannii complex which had reacted with the O-antigen-specific MABs and thus may indeed represent additional O serotypes within the respective Acinetobacter species. However, some of the patterns obtained after acid hydrolysis and immunostaining with MAB A6 were observed to be highly similar (Fig. 3). Patterns D and M, which were observed

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**FIG. 1.** Reactivities of LPSs (25 μg each) from Acinetobacter strains used for immunization (see Materials and Methods) after SDS-PAGE on a 10% resolving gel and immunostaining with homologous strains used for immunization (i.e., A. calcoaceticus and A. baumannii with MABs S51-3 and S53-32, respectively). The results described below were obtained in dot blots could be confirmed by performing Western blot analysis. Although in most cases the obtained ladder patterns were indistinguishable from the pattern obtained with the respective homologous LPS (data not shown), a different banding pattern was observed for a number of isolates when they were immunostained with MAB S48-13 or MAB S51-3 (Fig. 2). Comparison of the core-lipid A moieties of these isolates with that of the homologous strain in silver-stained SDS–15% polyacrylamide gels showed no difference in migration distance (data not shown), thus suggesting that the O antigens of these isolates are antigenically related but structurally distinct.

**FIG. 2.** Representative Western blots indicating differences in banding patterns observed between certain Acinetobacter isolates stained with MAB S48-13 (A) or S51-3 (B). Lanes 1, homologous strain; lanes 2, heterologous strain.
in some *A. baumannii* isolates, were found to be indistinguishable from patterns B and J, respectively, which were observed in some *Acinetobacter* genomic species 3 isolates. *A. baumannii* pattern E was found to be similar to pattern A, which was obtained with *Acinetobacter* genomic species 13TU isolates, and *A. baumannii* pattern A was indistinguishable from *A. calcoaceticus* pattern A.

**FIG. 3.** Representative O banding patterns observed for the species indicated below following acid hydrolysis of membrane-bound LPS after SDS-PAGE (10% resolving gel) of protease K-digested bacterial lysates and transfer onto a polyvinylidene difluoride membrane. (A) *A. calcoaceticus*; (B) *A. baumannii*; (C) *Acinetobacter* genomic species 3; (D) *Acinetobacter* genomic species 13TU.

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**DISCUSSION**

The potential of members of the genus *Acinetobacter* to cause infection has been known for decades (14, 15, 17). However, only after the improvement of species classification within the genus as a result of DNA-DNA hybridization studies (3, 4, 20, 32) was it possible to gain insight into the ecology and the clinical significance of individual *Acinetobacter* species. The increased recognition of members of the genus *Acinetobacter* as nosocomial pathogens (1, 33) has prompted numerous research groups to search for practical methods for the identification of *Acinetobacter* species which can be used reliably in clinical microbiology laboratories (4, 13). Due to the successful use of LPSs as taxonomic markers for a variety of gram-negative bacteria, we are currently investigating the possibility of an identification method for *Acinetobacter* strains, based on the O antigens of their LPSs. In the present study, a panel of five O-antigen-specific MAbs was generated against the LPSs of strains from species belonging to the *A. calcoaceticus-A. baumannii* complex and, together with three previously described MAbs, tested on a large collection of *Acinetobacter* strains isolated from various clinical and environmental sources in different geographical areas to determine the extent to which strains from the currently clinically important genomic species can be identified by these antibodies.

Reactivity was observed only with isolates from species comprising the *A. calcoaceticus-A. baumannii* complex. For two MAbs, reactivity was also observed with isolates belonging to species different from that of the strain which was used for generating the respective MAb. This observation supports an initial hypothesis (22) that O-antigenic determinants recognized by some antibodies may be present in more than one genomic group. With the generation of more O-antigen-specific MAbs in the future, it will be possible to clarify which determinants are specific for a given genomic group and which are not. So far, these are the only two MAbs which have shown cross-species reactivity.

Using a novel method to visualize any LPS via its lipid A moiety (the antigenicity of which is exposed only after acid hydrolysis) with antibody in Western blotting (21), it was possible to tentatively identify additional O serotypes among those isolates from the *A. calcoaceticus-A. baumannii* complex which had not reacted with any of the O antigen-specific MAbs used in this study. A total of 28 additional banding patterns were
observed. Although most of the banding patterns obtained after acid hydrolysis were distinguishable from each other and from the patterns observed for isolates from the *A. calcoaceticus-A. baumannii* complex which had reacted with the O antigen-specific MAbs, some patterns were found to be highly similar. Four of the banding patterns observed for *A. baumannii* isolates were indistinguishable from patterns observed for the other *Acinetobacter* species within the *A. calcoaceticus-A. baumannii* complex, namely, *A. calcoaceticus* (one pattern), *Acinetobacter* genomic species 3 (two patterns), and *Acinetobacter* genomic species 13TU (one pattern). Since the acid hydrolysis method does not give information as to the chemical compositions of the polysaccharide chains, it is uncertain whether these O antigens are indeed identical. Detailed biochemical and serological analyses of those polysaccharides which displayed similar banding patterns will be necessary to determine whether they are structurally and antigenically identical. However, those isolates from the *A. calcoaceticus-A. baumannii* complex which displayed a distinct banding pattern after acid hydrolysis have already provided a basis for future immunizations and screening strategies. The lack of detection of a banding pattern for all isolates (Table 1) which were subjected to the acid hydrolysis method has also been observed in previous studies (24, 25). The exact reason for this phenomenon is still uncertain but may be explained by these isolates having a severely reduced level of O-antigen expression, e.g., due to so far unidentified cultivation requirements, or by these isolates naturally producing LPS which does not contain an O antigen.

In this study, we have thus generated O-antigen-specific MAbs and shown that they are readily useful for the identification of strains belonging to species from the *A. calcoaceticus-A. baumannii* complex, which comprises those species which are of the utmost clinical importance within the genus *Acinetobacter* at present. As shown recently (23), such antibodies could be adapted to the format of a latex agglutination test for practical use in clinical microbiology laboratories. Although the MAbs generated in the present study do not cover all the isolates that were tested, we were able to delineate further putative O serotypes among species of the *A. calcoaceticus-A. baumannii* complex, which will be useful in choosing future immunization strategies aimed at isolating antibodies specific for these additional serotypes. Whether MAbs against other members of the genus *Acinetobacter* are indeed necessary is certainly questionable, since most species are thought to represent colonization rather than infection (10, 28, 31). Therefore, although our studies do not exclude the generation of antibodies against non-*A. calcoaceticus-A. baumannii* complex strains (26), they will continue to focus primarily on obtaining O-antigen-specific MAbs against the clinically important genomic groups, i.e., *A. baumannii*, *Acinetobacter* genomic species 3, and *Acinetobacter* genomic species 13TU, in order to fill current gaps. This will enable us to provide a complete O-serotyping scheme for these species, as has been successfully established for many other clinically significant gram-negative bacteria. Moreover, as has been reported in a recent study (27), such MAbs also have the potential to be employed as a means to define the prevalence of *Acinetobacter* serotypes in clinical settings and for the facile tracing of strains causing outbreaks in hospitals.

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