Characterization of epidemiologically unrelated Acinetobacter baumannii isolates from four continents by use of multilocus sequence typing, pulsed-field gel electrophoresis, and sequence-based typing of bla(OXA-51-like) genes

Citation for published version:

Digital Object Identifier (DOI):
10.1128/JCM.02431-09

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Journal of Clinical Microbiology

Publisher Rights Statement:
http://journals.asm.org/site/misc/ASM_Author_Statement.xhtml

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Characterization of Epidemiologically Unrelated Acinetobacter baumannii Isolates from Four Continents by Use of Multilocus Sequence Typing, Pulsed-Field Gel Electrophoresis, and Sequence-Based Typing of blaOXA-51-like Genes

Ahmed Hamouda, Benjamin A. Evans, Kevin J. Towner and Sebastian G. B. Amyes


Updated information and services can be found at:
http://jcm.asm.org/content/48/7/2476

These include:

**REFERENCES**
This article cites 48 articles, 27 of which can be accessed free at: http://jcm.asm.org/content/48/7/2476#ref-list-1

**CONTENT ALERTS**
Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to: http://journals.asm.org/site/subscriptions/
Characterization of Epidemiologically Unrelated *Acinetobacter baumannii* Isolates from Four Continents by Use of Multilocus Sequence Typing, Pulsed-Field Gel Electrophoresis, and Sequence-Based Typing of \(\text{bla}_{\text{OXA-51-like}}\) Genes

Ahmed Hamouda,1* Benjamin A. Evans,1 Kevin J. Towner,2 and Sebastian G. B. Amyes1

Centre for Infectious Diseases, University of Edinburgh, Edinburgh, United Kingdom,1 and Nottingham University Hospitals NHS Trust, Nottingham, United Kingdom2

Accepted 20 April 2010

This study used a diverse collection of epidemiologically unrelated *Acinetobacter baumannii* isolates to compare the robustness of a multilocus sequence typing (MLST) scheme, based on conserved regions of seven housekeeping genes, *gltA*, *gdhB*, *recA*, *cpn60*, *rpoD*, *gyrB*, and *gpi*, with that of sequence-based typing of *\(\text{bla}_{\text{OXA-51-like}}\)* genes (SBT-*\(\text{bla}_{\text{OXA-51-like}}\)* genes). The data obtained by analysis of MLST and SBT-*\(\text{bla}_{\text{OXA-51-like}}\)* genes were compared to the data generated by pulsed-field gel electrophoresis (PFGE). The topologies of the phylogenetic trees generated for the *gyrB* and *gpi* genes showed evidence of recombination and were inconsistent with those of the trees generated for the other five genes. MLST identified 24 sequence types (STs), of which 19 were novel, and 5 novel alleles. Clonality was demonstrated by eBURST analysis and standardized index of association values of >1 (\(P < 0.001\)). MLST data revealed that all isolates harboring the major *\(\text{bla}_{\text{OXA-51-like}}\)* alleles *OXA-66*, *OXA-69*, and *OXA-71* fell within the three major European clonal lineages. However, the MLST data were not always in concordance with the PFGE data, and some isolates containing the same *\(\text{bla}_{\text{OXA-51-like}}\)* allele demonstrated <50% relatedness by PFGE. It was concluded that the *gyrB* and *gpi* genes are not good candidates for use in MLST analysis and that a SBT-*\(\text{bla}_{\text{OXA-51-like}}\)* gene scheme produced results comparable to those produced by MLST for the identification of the major epidemic lineages, with the advantage of a significantly reduced sequencing cost and time. It is proposed that studies of *A. baumannii* epidemiology could involve initial screening of *\(\text{bla}_{\text{OXA-51-like}}\)* alleles to identify isolates belonging to major epidemic lineages, followed by MLST analysis to categorize isolates from common lineages, with PFGE being reserved for fine-scale typing.

*Acinetobacter baumannii* is a Gram-negative bacterium that causes serious nosocomial infections, especially in critical care units (13, 14, 42). Several outbreaks have been caused by multidrug-resistant (MDR) strains of *A. baumannii* (23, 33, 43), and the rate of resistance to carbapenems, which have been the antibiotics of choice to treat infections caused by this pathogen, has increased considerably over the last decade (4, 32, 42, 46). In addition, the prevalence of *A. baumannii* in hospitals has increased worldwide (3, 25, 28, 29, 47), and, therefore, finding suitable molecular typing methods for *A. baumannii* is essential for epidemiological investigations and infection control studies. Many genomic typing methods have been used, including ribotyping (36), infrequent-restriction-site analysis (48), repetitive extragenic palindromic sequence-based PCR (rep-PCR) (18), random amplified polymorphic DNA (RAPD) analysis (21), amplified fragment length polymorphism (AFLP) analysis (39), and multilocus PCR and electrospray ionization mass spectrometry (PCR/ESI-MS) (6). Pulsed-field gel electrophoresis (PFGE) is still considered the “gold standard” for the typing of bacterial isolates (36), but it has drawbacks when its comes to interchanging data among laboratories for comparison purposes (35) and may lose its discriminatory power when isolates from geographically diverse areas are analyzed.

Multilocus sequence typing (MLST) schemes, which use several housekeeping genes, have already been used to type many pathogenic bacteria (15, 17, 20, 40), including *A. baumannii* (1, 31, 44), and MLST is emerging as an alternative to PFGE. MLST is used mainly for global epidemiology studies, but it has also been used successfully for short-term investigation of an outbreak of meningococcal disease (11). Although MLST has many advantages over other molecular typing methods, many questions remain to be answered, including whether several loci are required to obtain a robust scheme and whether the criteria for the selection of the housekeeping genes are sufficiently reliable to reveal the population structure of the strains analyzed.

*\(\text{bla}_{\text{OXA-51-like}}\)* genes are unique to *A. baumannii* and may be used as markers for identification of this species (16). They have also successfully been used as one of three loci in a PCR-based typing scheme that is able to assign isolates of *A. baumannii* to sequence groups (SGs) that appear to correlate with the major epidemic lineages within the species (41). This raises the question of whether the *\(\text{bla}_{\text{OXA-51-like}}\)* genes themselves could be utilized in a typing scheme. Accordingly, the aim of this work was to investigate the robustness of MLST in categorizing epidemiologically unrelated *A. baumannii* isolates from four continents and to evalu-
ulate the use of variations within the intrinsic \textit{bla}_{OXA-51-ble} gene as a typing tool comparable to MLST.

**MATERIALS AND METHODS**

**Bacterial isolates.** Forty-four isolates of \textit{A. baumannii} from 22 countries, including three standard representatives of the endemic pan-European clonal lineages I, II, and III, were included in this study. All isolates were identified as \textit{A. baumannii} as described previously (8) and by the presence of a \textit{bla}_{OXA-51-ble} gene (45). All the isolates were recovered from cases of invasive disease in various hospitals between 1982 and 2006.

**PFGE.** PFGE was performed essentially as described previously (27), but with minor modifications. The agarose-embedded bacterial genomic DNA was digested with Apal (Promega, Southampton, United Kingdom) at 37°C overnight. Electrophoresis was performed in both agarose (1%, w/vol) gels with 0.5X Tris-borate-EDTA buffer. The following PFGE parameters were applied: voltage of 6 V/cm, initial switch time of 5 s, final switch time of 35 s, and run time of 24 h. The stained gels were scanned using the Diversity Database software image-capturing system (Bio-Rad). The Dice coefficient was used to calculate similarities, and the tree topologies were conducted visually, and differences were con-

**MLST analysis.** DNA was purified using a Puregene DNA purification system (Genta Systems, Minneapolis, MN). PCRs for the seven housekeeping genes, \textit{glaA}, \textit{gldB}, \textit{gldhB}, \textit{recA}, \textit{cpn60}, \textit{gpi}, and \textit{rpoD}, were performed in 50-μl volumes containing 10 μl 5X Green GoTaq Flexi buffer, 1.5 mM MgCl$_2$, 800 nM PCR nucleotide mix, and 1.25 U GoTaq DNA polymerase (Promega). The primers and PCRs conditions were those described by Bartual et al. (1), except that the annealing temperatures were modified to 45°C for \textit{gldB}, \textit{gldhB}, \textit{recA}, and \textit{rpoD}, to 56°C for \textit{gof}, \textit{S}, \textit{I} A, and 1,000 iterations.

**Sequence-based typing of \textit{bla}_{OXA-51-ble} genes (SBT-\textit{bla}_{OXA-51-ble} genes).** Genomic DNA was extracted by boiling two to three colonies for 10 min in 50 μl sterile distilled water. The PCR mixtures contained 20 μM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 2 mM MgSO$_4$, nuclease-free bovine serum albumin (0.1 mg/ml), 0.1% Triton X-100, 1.5 mM MgCl$_2$, 800 μM PCR nucleotide mix, and 1.25 U of \textit{Pfu} DNA polymerase (Promega) in a total volume of 50 μl. Primers \textit{OXA-69A} and \textit{OXA-69B} (16), external to the \textit{bla}_{OXA-51-ble} gene, were used to amplify the entire sequence under the following conditions: 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 48°C for 40 s, and 72°C for 3 min and then 72°C for 6 min. Primers were used at a final concentration of 0.25 μM, and reactions were performed with 0.5 μl crude DNA template. PCR product analysis and sequencing were performed as described above for the MLST products.

**RESULTS**

**PFGE.** Six small clusters of isolates, each with a similarity of >80%, were identified (Fig. 1). The isolates forming each cluster were considered to belong to the same epidemic lineage. The remaining isolates had PFGE profiles with similarities of <80% and were considered to be unrelated.

**MLST analysis.** Seven housekeeping genes, \textit{glaA}, \textit{gldB}, \textit{recA}, \textit{cpn60}, \textit{rpoD}, \textit{gyp}, and \textit{gpi}, were amplified and sequenced for each isolate. Twenty-four different STs were identified, of which 19 (designated ST33 to ST51) were novel, and five novel alleles, officially named \textit{gdhB21}, \textit{gdhB22}, \textit{recA16}, \textit{rpoD22}, and \textit{gpi26}, were identified (Fig. 1). (i) Tree congruence. Comparison of the tree topologies for all identified alleles of the seven genes revealed inconsistencies with the trees for \textit{gyp} and \textit{gpi} in relation to one another and to the other five trees. The topologies of the trees for the \textit{glaA}, \textit{cpn60}, \textit{gldB}, \textit{ recA}, and \textit{rpoD} sequences were broadly consistent, with the same isolates being grouped together in the same major clades with few exceptions. However, the \textit{gpi} tree splits the 12 isolates usually grouped with isolates A297 (the representative of European clone I) across two major clades, while the \textit{gyp} tree splits the 17 isolates usually grouped with isolate A320 (the representative of European clone II) across two major clades. This observation was confirmed via the quartet measure of tree-to-tree distances (7). This method measures the similarity between two trees by breaking them down into quartets, subtrees containing just four isolates. By analyzing whether all possible quartets across both trees are resolved identically to or differently from one another, it can be determined whether the topologies of the two trees are similar or not. For the \textit{gyp} and \textit{gpi} trees, the number of differently resolved trees was greater than the number of similarly resolved trees, in contrast to the results for the other five genes (Table 1), confirming the visual observation that the topologies of these two trees are inconsistent with those of the other five genes.

(ii) PHI test. The PHI test utilizes DNA sequence data and infers whether patterns of nucleotide polymorphisms are consistent with a model of vertical transmission (clonal population structure) or not. Instances where they are not may indicate recombination events (2). Analysis of alleles for all seven genes with the PHI test detected statistically significant ($P = 0.0036$) evidence for recombination within the alleles for \textit{gyp} but not in \textit{gpi}.

(iii) eBURST analysis. eBURST analysis of the allelic profiles for all seven loci by the use of both stringent and relaxed grouping parameters produced the same result (Fig. 2A), re-
FIG. 1. Dendrogram constructed following determination of PFGE profiles using UPGMA. The OXA-51 type, the ST, and the MLST allelic profiles of seven housekeeping genes are shown for each isolate. ID, isolate identifier; ´, novel allelic profile; *, novel sequence.
revealing four CCs and nine singletons. The largest clonal complex, CC1, contained ST34, which was identified as a potential founder, with ST46, ST36, ST40, ST4, and ST22 radiating from it. A second major clonal complex, CC2, contained ST49, ST51, ST47, and ST25 surrounding ST33, which was identified as a potential founder for the complex. The other two minor clonal complexes identified contained ST35 and ST38 (CC3) and ST51, ST47, and ST25 surrounding ST33, which was identified as a potential founder, with ST46, ST36, ST40, ST4, and ST22 radiating from it. The majority of isolates fell into one of three distinct and highly related clades: 14 isolates were grouped with the European clone II strain, 12 isolates were grouped with the European clone I strain, and 4 isolates were grouped with the European clone III strain. There was complete agreement between the CC groupings by eBURST analysis and the clades in the five-locus tree.

**SBT-bla** _OXA-51-like_ genes. Genes for 12 different _OXA-51-like_ enzymes were identified, of which _OXA-66 (n = 11)_ , _OXA-69 (n = 6)_ , and _OXA-71 (n = 5)_ constituted three major groups (Fig. 1). _OXA-51_ and _OXA-107_ were each found in three isolates, while _OXA-83_ was found in two isolates. _OXA-68, OXA-82, OXA-92, OXA-109_, and _OXA-112_ were found in a single isolate each (Fig. 1). As reported previously (8), representatives of the three major European clonal lineages were found to encode enzymes representative of the major groups, with clone I encoding _OXA-69, clone II encoding _OXA-66_, and clone III encoding _OXA-71_.

**Evaluation of SBT-bla** _OXA-51-like_ gene data and its correlation with MLST and PFGE data. The _SBT-bla_ _OXA-51-like_ gene data correlated well with the MLST data, with respect to the identification of the major European lineages. The 14 isolates that grouped with the European clone II isolate each encoded closely related _OXA-51-like_ enzymes of the _OXA-66_ group (Fig. 3A). Similarly, the 12 isolates that grouped with the European clone I isolate each encoded closely related members of the _OXA-69_ group of _OXA-51-like_ enzymes, while the 4 isolates that grouped with the European clone III isolate each encoded _OXA-71_. A few exceptions were noticed in which isolate STs and _OXA_ gene sequences were not consistent. Isolates A187 and A483, which had the same ST, ST20, encoded different _OXAs, OXA-68_ and _OXA-51_ (8 amino acid differences); and isolate A92, which had an _OXA-69_ enzyme, was not grouped with the other _OXA-69-encoding_ isolates. However, the _bla_ _OXA-69_ gene in A92 contained 5 silent nucleotide substitutions (G426 → A, C474 → A, C511 → T, G540 → A, and T801 → C), suggesting that this isolate may be quite different from the other _OXA-69-encoding_ isolates. For the three major epidemic lineages, the _SBT-bla_ _OXA-51-like_ gene data were in concordance with the MLST-derived phylogeny for 93% of the isolates related to European clone I, 94% of the isolates related to European clone II, and 100% of the isolates related to European clone III. In contrast, typing by PFGE was not always in concordance with the _SBT-bla_ _OXA-51-like_ gene data or the MLST data. There were isolates with the same PFGE type (types A4, A6, and A24) that shared the same _OXA-51-like_ gene, whereas others had the same ST but a different PFGE type (e.g., isolates A25 and A4 and isolates A6 and A24) (Fig. 1).

**DISCUSSION**

_A. baumannii_ is becoming one of the most problematic organisms currently responsible for nosocomial infections, espe-
cially in intensive care units (9). Increasing antimicrobial resistance (42) and the ability of *A. baumannii* to survive on inanimate and dry surfaces (19) have been linked to the occurrence of outbreaks observed in various hospitals (5). MLST has emerged as the technique of choice for studying the population structure of many bacterial species (15, 17, 20, 30, 40), including *A. baumannii* (1, 10, 24, 29, 37, 38). However, profiles for only 93 isolates of *A. baumannii* are available in the current MLST database (http://pubmlst.org/abaumannii/), which is considerably less than the numbers deposited in databases for other bacteria (http://pubmlst.org/databases.shtml). Therefore, in order to investigate the MLST scheme thoroughly and validate the robustness of the MLST scheme, the present study investigated a previously characterized and diverse collection of epidemiologically unrelated *A. baumannii* isolates collected from 22 countries over four continents, Europe, Asia, Africa, and South America (8). In addition, because *bla*<sub>OXA-51-like</sub> genes are endogenous to *A. baumannii*, the study aimed to evaluate the use of these genes as the basis for a typing scheme.

The diversity of the isolates used in this study, compared with the limited diversity of the isolates in the *A. baumannii* database, meant that a considerable number of novel STs was expected. Indeed, 19 novel STs were detected, with this result being similar to that of Wisplinghoff et al. (44). Together with some earlier preliminary supporting evidence (1, 44), this indicates that *A. baumannii* is more diverse than originally thought, especially as *A. baumannii* tends to spread clonally during outbreaks (5, 34). Although MLST has advantages over

FIG. 2. Results of eBURST analysis used to assign CCs within the 44 isolates of *A. baumannii* utilizing seven loci (A) and five loci (B). (A) The CCs are circled, and the predicted clonal ancestors for CC1 and CC2 are shown by the central points. (B) ST33 encompasses ST33, ST37, ST49, ST51, and ST47; ST34 encompasses ST34, ST4, ST36, ST40, ST46, and ST22; and ST35 includes ST35 and ST38, while ST48 covers ST48 and ST50. The sizes of the points are proportional to the number of isolates assigned to each ST.
FIG. 3. Neighbor-joining phylogenetic tree based on seven (A) and five (B) housekeeping genes. The corresponding OXA-51-like enzymes that the isolates encode and their sequence types are shown beside each isolate number. The CC that isolates were assigned to by eBURST analysis is indicated. Branches are labeled with percent support. OXA-69*, the OXA-69 enzyme encoded by a blaOXA-69 gene containing 5 silent nucleotide substitutions.
many conventional methods for strain genotyping, it is not yet clear whether phylogenetic relationships based on several, often randomly selected, loci reflect the real phylogenies of the strains investigated or whether a smaller number of loci could provide results similar to those based on several loci (22).

The present work revealed that the tree topologies for all identified alleles of the seven genes were inconsistent, with the trees for gyrB and gpi being incongruent in relation to each other and to the trees for the other five genes. The incongruence observed between gene trees suggests that horizontal gene transfer has occurred at the gyrB and gpi loci with a sufficient frequency that their inclusion in the estimation of the A. baumannii phylogeny would distort it to such a degree that it would not be representative of the phylogenetic structure of the core genome. The only previous study (31) to have published the results of an in-depth analysis using the MLST scheme of Bartual et al. (1) appears to have had the same problems encountered here, with the trees for gyrB and gpi being nonconcordant with those for the other loci.

By omitting the gyrB and gpi genes and using only the remaining five genes, a well-supported phylogenetic tree was obtained. This poses two questions: (i) can fewer genes be utilized as effectively to estimate a phylogeny representative of the diversity of the core genome, and (ii) what are the criteria, if any, used to select the best housekeeping genes and/or other genes to reflect the core genome diversity? The present study revealed that only five genes (gltA, gdhB, recA, cpn60, and rpoD) were required to characterize A. baumannii and that these five genes were able to provide sufficient discriminatory power to separate the three major European clonal lineages, lineages I, II, and III, into three different groups. Furthermore, compared to MLST, SBT-bla
toX51-like
genes successfully identified 94% of these isolates as belonging to a major epidemiological lineage, with only two results being discrepant. Members of the three major European lineages are responsible for the majority of outbreaks caused by A. baumannii worldwide and, as such, are currently of greater concern from an infection control perspective than other unrelated lineages belonging to this species. Therefore, it is very useful that isolates can quickly and easily be identified as belonging to one of the three major European epidemic lineages with a high degree of accuracy by SBT-bla
toX51-like
genes.

The $I^s$ value for the entire data set was significantly greater than 0, indicating that the population structure is clonal. However, when only one representative of each ST was included in the analysis, the $I^s$ value, while it was still significantly greater than 0, decreased from 0.4907 to 0.3569. This decrease is concordant with the epidemic nature of the European clonal lineages predominant in the data set, though it may also point toward there being a greater degree of recombination within more closely related lineages of A. baumannii. Identification of a clonal population structure by $I^s$ analysis seems at odds with the detection of recombination at the gyrB and gpi loci. However, it has previously been shown that when $I^s$ measures are used, the presence of recombination may be masked either due to an epidemic population structure or due to recombination occurring only between closely related lineages and not with those more distantly related. Analysis of a larger data set is required to determine which scenario is applicable within A. baumannii.

In contrast to a previous study (1), the PFGE profiles did not always correlate with the MLST results (Fig. 1). Therefore, it is probable that the swift increase in the genetic diversity indexed by PFGE has led to significant discrepancies in the Apal DNA fragment patterns, such that these isolates are no longer recognized as being related to one another by the criteria utilized here.

With the exclusion of the gyrB and gpi loci, MLST analysis separated the representative isolates of European clones I (ST33), II (ST34), and III (ST35) and the isolates associated with these lineages into three major groups. Isolates A63 (ST33) and A479 (ST47), which grouped with clone I, are closely related (the two STs are single-locus variants at the gpi locus) and were recovered from Buenos Aires, Argentina, and Pakistan, respectively. Similarly, isolate A401 (ST22) from Taiwan grouped with clone II, ST34, though it differs at the gyrB and gpi loci. Thus, both of these clonal lineages are not confined solely to Europe. This is in agreement with other studies of worldwide A. baumannii isolates. The present study detected isolates belonging to the European clone III lineage only in European cities, though this does not preclude their spread and/or presence in other parts of the world.

Overall, typing of isolates by their bla
toX51-like
gene sequence yielded results that were broadly consistent with those obtained by MLST, with few exceptions. The data were considerably more consistent with the MLST data than with the PFGE data. The enzymes OXA-66, OXA-69, and OXA-71 are the predominant members of closely related OXA-51-like subgroups and are associated with particular epidemic lineages (8). Both SBT-bla
toX51-like
genes and MLST grouped the epidemic European clones I, II, and III into three different lineages. MLST shows clear applications for studying evolutionary relationships on a global scale and is the best state-of-the-art technique available to study population structures. However, the present data show that the gyrB and gpi loci should be treated with caution when the phylogeny of the core genome is estimated, particularly with respect to assigning isolates to the major epidemic lineages. Furthermore, the SBT-bla
toX51-like
gene scheme was found to be a useful tool for accurately identifying isolates belonging to the three major epidemic lineages within A. baumannii.

ACKNOWLEDGMENT

This work was supported by a grant (grant RA0119) from the Medical Research Council of the United Kingdom.

REFERENCES


