Genes encoding ten newly designated OXA-63 Group class D β-lactamases identified in strains of the pathogenic intestinal spirochaete *Brachyspira pilosicoli*.

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Abstract

The anaerobic spirochaete *Brachyspira pilosicoli* colonises the large intestine of birds and mammals, including human beings, and may induce colitis and diarrhoea. *B. pilosicoli* has a recombinant population structure, and strains show extensive genomic rearrangements and different genome sizes. The resident chromosomal gene *bla*~\textsubscript{OXA-63}~ in *B. pilosicoli* encodes OXA-63, a narrow spectrum group IV class D β-lactamase. Genes encoding four OXA-63 variants have been described in *B. pilosicoli*, and the current study was designed to investigate the distribution and diversity of such genes and proteins in strains of *B. pilosicoli*. PCRs were used to amplify *bla*~\textsubscript{OXA-63}~ group genes from 118 *B. pilosicoli* strains from different host species and geographical origins. One primer set was targeted externally to the gene and two sets were designed to amplify internal components. Sixteen strains (13.6%) showed no evidence of possessing *bla*~\textsubscript{OXA-63}~ group genes, 44 (37.3%) had a full gene, 27 (22.9%) apparently had a gene but it failed to amplify with external primers, and 29 (24.6%) had only one or other of the two internal components amplified. Based on translation of the nucleotide sequences, ten new variants of the β-lactamase, designated OXA-470 through OXA-479 were identified amongst the 44 strains that had the full gene amplified. The 16 strains lacking *bla*~\textsubscript{OXA-63}~ group genes had a region of 1674 base pairs missing around where the gene was expected to reside. Despite apparent genomic rearrangements occurring in *B. pilosicoli*, positive selection pressures for conservation of *bla*~\textsubscript{OXA-63}~ group genes and OXA proteins appear to have been exerted.
Introduction

*Brachyspira pilosicoli* is an anaerobic intestinal spirochaete that can colonise the large intestine of many host species. The spirochaete has a recombinant population structure (Trott *et al*., 1998; Neo *et al*., 2013), and genomic sequencing has revealed that strains show extensive genomic rearrangements and that their genome sizes vary (Wanchanthuek *et al*., 2010; Mappley *et al*., 2012; Lin *et al*., 2013). Infection with *B. pilosicoli* occurs commonly in intensively housed pigs and adult poultry, and also in human beings living in crowded conditions in developing countries (Trott *et al*., 1997; Mikosza & Hampson, 2001; Margawani *et al*., 2004). There are experimental and epidemiological data showing that *B. pilosicoli* strains can be transmitted between host species and cause disease (Trott *et al*., 1996; Hampson *et al*., 2006).

OXA-63 is a narrow spectrum group IV class D β-lactamase encoded by the gene *bla*<sub>OXA-63</sub> that was first described in *B. pilosicoli* strain BM4442 isolated from a human being in France (Meziane-Cherif *et al*., 2008). This enzyme contributes to penicillin resistance in the spirochaete, and in particular confers resistance to oxacillin, benzylpenicillin and ampicillin by cleaving the β-lactam ring (Meziane-Cherif *et al*., 2008). Following the original description of the gene, three variants (*bla*<sub>OXA-136</sub>, *bla*<sub>OXA-137</sub> and *bla*<sub>OXA-197</sub>) have been described in other strains of *B. pilosicoli* isolated from humans, pigs and birds (Mortimer-Jones *et al*., 2008; Jansson & Pringle, 2011). These enzymes are unique to *B. pilosicoli* and constitute the “OXA-63 group”, with the nearest neighbour being OXA-85 from *Fusobacterium nucleatum* subsp. *polymorphum* (Voha *et al*., 2006; Poirel *et al*., 2010). In *B. pilosicoli* strains BM4442 and 95/1000 the genes are not associated with the local presence of integrons, insertion sequences or transposons, hence supporting it being a resident gene rather than being acquired by recent lateral transfer (Poirel *et al*., 2010). The low GC ratio of the gene resembles that of the rest of the genome, also implying that it has not been recently acquired. *B. pilosicoli* strains that
apparently lack bla\textsubscript{OXA-63} group genes have been identified (Brooke et al., 2003; Mortimer-Jones et al., 2008).

The purpose of this study was to investigate the distribution of bla\textsubscript{OXA-63} group genes and OXA proteins in strains of \textit{B. pilosicoli} from different sources, with the object of gaining a better understanding of their diversity and how the recombinant population structure may have impacted on these resident chromosomal genes.

**Materials and Methods**

\textit{Brachyspira pilosicoli} strains and culture. A total of 117 \textit{B. pilosicoli} strains were obtained from the culture collection held at Murdoch University (Table 1). They represented a range of sequence types (STs) previously identified in multilocus sequence typing (MLST) (Neo et al., 2013), and were isolated between 1978 and 2004 from different geographical regions and species. They originated from different States of Australia (n=64), from Papua New Guinea (n=29), the United States of America (n=8), Canada (n=5), Italy (n=5), the United Kingdom (n=3), France (n=2) and New Zealand (n=1). The collection included 46 isolates recovered from pigs, 41 from human beings, 23 from chickens, five from dogs and two from horses.

The strains were thawed and cultured on Trypticase Soy agar (TSA) plates containing 5% defibrinated ovine blood for five days at 37°C in an anaerobic jar with an atmosphere of 94% H\textsubscript{2} and 6% CO\textsubscript{2} generated using a GasPak Plus gas generator envelope (BBL). A zone of weak haemolysis around the inoculated culture indicated growth, and confirmation was obtained by re-suspending surface growth in phosphate buffered saline (PBS) and looking for the presence of motile spirochaetes using a phase-contrast microscope. Species identity was confirmed by
using a PCR reaction targeting the 16S rRNA gene (La et al., 2003). Subcultures were made into pre-reduced anaerobic Trypticase Soy broth (Kunkle et al., 1986).

**Analysis of whole genome sequences.** The genome sequences of 36 strains of nine *Brachyspira* species, either downloaded from the NCBI database or as unpublished sequences from our laboratory were examined for * bla*<sub>OXA-63</sub> group genes using the BlastN function of the Geneious R7 software (Biomatters Ltd). They included the sequences of the four *B. pilosicoli* strains 95/1000, WesB, B2904 and P43/6/78<sup>T</sup> (accession numbers NC_014330, NC_018604, NC_018607 and NC_019908, respectively), 20 *B. hyodysenteriae* strains (accession numbers NC_012225 for the chromosome and NC_012226 for the plasmid of WA1, and NZ_JXNAOOOOOOOO through NZ_JXNSOOOOOOOO for the other 19 strains: Black et al., 2015), five “*B. hampsonii*” strains (accession numbers NZ_ALNZ00000000 and NZ_AOMM00000000, and three unpublished), two *B. intermedia* strains (NC_017243 and one unpublished), one *B. innocens* strain (NZ_ARQI00000000), one *B. murdochii* strain (NC_014150), one *B. alvinipulli* strain (NZ_JADF00000000), one *B. aalborgi* strain (unpublished) and one “*B. suanatina*” strain (NZ_CVLB00000000).

**Polymerase chain reaction for the β-lactamase gene.** For each *B. pilosicoli* strain, 10ml of broth containing ~10<sup>8</sup> cells/ml was centrifuged at 5000g. The supernatant was discarded and the pellet re-suspended in an equal volume of PBS (pH 7.4) and heated at 95°C for 15 min to release the DNA, before storing it at -20°C. The solution containing the extracted DNA was used as the template for the PCR reactions.

Three PCR primer sets (Bp-oxa_U/D, Bp-lac1-F/R and Bp-oxa-F/R) were developed for amplification of * bla*<sub>OXA-63</sub> group genes (Table 2; Figure 1). These were based on the sequence obtained from the French human strain BM4442 and from the sequenced Australian porcine.
strain 95/1000 (Wanchanthuek et al., 2010). The primers Bp-oxa U/D were targeted outside the gene and the other two pairs were internal. The PCR mixture consisted of 1x PCR buffer, 1.5mM MgCl2, 0.5U Taq DNA polymerase, 0.2mM of each dNTP, 0.5µM of forward and reverse primer and template. Cycling conditions involved an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and primer extension at 72 °C for 2 min (Mortimer-Jones et al., 2008). The products were separated by gel electrophoresis and visualized after staining with ethidium bromide.

**Sequencing of bla\textsubscript{OXA-63} group genes.** Forty-four strains that produced products with all three of the primer pairs were chosen for the sequencing of the \textit{bla}_{OXA-63} group genes, using the ABI 373A sequencing system (Applied Biosystems). The sequenced areas were aligned and combined into a single sequence of the \textit{bla}_{OXA-63} group gene. These sequences were translated to the predicted amino acid sequences of the enzymes in the strains using the MEGA4 software by matching the complementary triplet codon. The results then were aligned with the predicted amino acid sequences from \textit{B. pilosicoli} strains B4442 and 95/1000 using ClustalW and the Bioedit Sequence Alignment Editor (Hall, 1999).

All nucleotide sequences were placed in a single FASTA formatted file, aligned with ClustalW and edited with the \textit{bla}_{OXA-63} gene to the targeted length, before being converted to the MEGA format (http://ccg.murdoch.edu.au/tools/clustalw2mega/). Trees were constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method for the aligned DNA sequences and the predicted translated amino acid sequences using the MEGA v4.0.2 program (Tamura et al., 2001). The nucleotide and predicted amino acid sequences were subjected to comparison with known β-lactamase genes and proteins using the NCBI Blast website. Predicted new proteins that varied by one or more amino acids from the known OXA proteins were assigned new OXA designations.
PCR and sequencing in strains lacking \textit{bla}_{OXA-63} group genes. For the 16 strains where a gene was not detected using the original three primer pairs, a region of 495 bp predicted to span across the site of the missing gene from base position 1,752,070 to 1,752,565 in strain P43/6/18$^T$ (lacking a \textit{bla}_{OXA} group gene) was targeted for amplification using primers P43-US and P43-DS (Table 2). The PCR products were sequenced as for \textit{bla}_{OXA-63} group genes.

\textbf{β-Lactamase production.} A total of 57 strains (44 with \textit{bla}_{OXA-63} group genes and 13 lacking the genes) were assessed for β-lactamase production by using BBL Cefinase nitrocefin disks (Becton Dickinson) (O'Callaghan \textit{et al.}, 1972). Each disk was moistened with sterile distilled water and a microbiology loop of spirochaete growth was smeared onto the disk surface. The disk was protected from drying and examined for a colour change reaction (from colourless to red) within 10 minutes and then at 30 minutes. \textit{B. pilosicoli} 95/1000 and P43/6/78$^T$ were included as known positive and negative controls, respectively.

\textbf{Antimicrobial susceptibility testing.} The same 57 strains of \textit{B. pilosicoli} were tested for susceptibility to ampicillin. The minimum inhibitory concentration (MIC) was determined using doubling dilutions of antibiotic from 1 to 128μg ml$^{-1}$ in an agar dilution method (Brooke \textit{et al.}, 2003). The test plates consisted of Trypticase Soy agar containing 5% defibrinated ovine blood and the appropriate antibiotic concentration. No ampicillin was added to the control plates.

Inocula were prepared by resuspending the growth of \textit{B. pilosicoli} from the surface of a blood agar plate in 1 ml of sterile PBS. They were counted using a haemocytometer chamber viewed under a phase-contrast microscope, with further dilution being made in PBS before $10^5$ cells of each strain were stab-inoculated onto test and control plates in triplicate. The plates were
incubated for 8 days at 37°C in an anaerobic jar, with an atmosphere of 94% H₂ and 6% CO₂ and then observed for haemolysis around the stab marks. The first sensitive colony zone and the last resistant colonies were resuspended in PBS and checked for spirochaete growth using a phase contrast microscope at a magnification of x400. The MIC was recorded as the lowest concentration of ampicillin that inhibited growth. An MIC of 32 or more was used as being indicative of lack of susceptibility (Jansson & Pringle, 2011).

Results

Genome comparisons

No bla OXA genes were found in the 32 non- B. pilosicoli genome sequences that were examined.

In B. pilosicoli strain 95/1000 the 807 base pair (bp) bla OXA-63 group gene was at position 1,472,303 to 1,473,109 adjacent to a tRNA-(guanine-N(7))-methyltransferase and a hypothetical protein. In strain WesB the gene was at position 161,378 to 162,184 and had the same adjacent genes as 95/1000. In strain B2904 the gene was at position 2,646,462 to 2,647,268 and was adjacent to two transposases and a hypothetical protein. Bla OXA-63 group genes were not found in P43/6/78 T, and sequence comparison with the 95/1000 and WesB genomes revealed that a 1,674 bp region starting 299 bp upstream and extending seven bp downstream from the bla OXA-63 group gene was missing from the P43/6/78 T genome. No transposases were identified in P43/6/78 T (Fig. 1). Strain B2904 had 34 transposases, WesB had 10, and 95/1000 had one gene encoding a possible transposase-like protein.

PCR and sequencing

Sixteen (13.6%) strains that were positive in the B. pilosicoli 16S rRNA PCR did not yield any products in the PCRs for bla OXA-63 group genes. Their identities are shown in Table 1 and they originated from human beings, chickens and pigs, and notably nine were from humans or pigs.
living the Asaro valley in the Eastern Highlands of Papua New Guinea (Trott et al., 1997). The strains came from a diverse array of STs in MLST, but some were closely related in adjacent STs. PCR analysis of this region identified that in all strains a product of approximately 495 bp was produced using the P43-US/DS primers compared to a product of approximately 1,674 bp for 95/1000. Sequencing of the 495 bp PCR product showed that this region was conserved in all 16 strains, with nucleotide similarity between 97.1% and 100%.

The other 102 (86.4%) strains tested had evidence for all or part of \( bla_{OXA-63} \) group genes being present; however, only 44 produced products in all three PCR reactions, and these were used for comparison of the sequence of the \( bla_{OXA-63} \) group genes. Another 27 (22.9%) strains amplified with the two internal primer sets but not with the external primer set (Bp-oxa-US/DS), 21 (17.8%) amplified only with internal primer set Bp-oxa-F484/R692 and eight (6.8%) only amplified with internal primer set Bp-lac1-F192/R634.

In all but strain OF15 the size of the \( bla_{OXA-63} \) group genes varied from the 804 bp size of the original \( bla_{OXA-63} \) gene of strain BM4442. The genes in the other 43 strains were 807 bp, which increased their predicted protein size by one amino acid. The \( bla_{OXA-63} \) group genes all showed relatively minor variation (six or fewer nucleotides difference) from the 807 bp \( bla_{OXA-136} \) in reference strain 95/1000. The phylogenetic relationship amongst the genes in the 44 strains and the known \( B. pilosicoli \) \( bla_{OXA-63} \) group genes obtained from GenBank is reflected in the UPGMA tree created using the nucleotide sequences (Fig. 2). As previously reported, the genes were all distinct from other \( bla_{OXA} \) genes that have been described in other bacterial species (Poirel et al., 2010).

**OXA-63 group variants**
The results for the translated amino acid sequence in the 44 strains that were compiled and aligned are shown in Figure 3. The predicted OXA-63 group proteins in the strains varied from 268 to 269 amino acids. All five of the highly conserved active-site elements of class D β-lactamases were predicted to be present in the proteins from the 44 strains. As the OXA enzymes all had more than 80% amino acid identity they all belonged to the OXA-63 group (Poirel et al., 2010). The protein in strain Gap51.2 that previously had been designated as OXA-136 was distinct from OXA-136 in strains 95/1000, COF-10 and WesB. Based on there being at least one new amino acid in the protein from Gap51.2, this was re-designated as OXA-471 (http://www.lahey.org). In addition nine other new OXA proteins were identified (OXA-470; and OXA-472 through OXA-149 table 1). Their relationships are shown in figure 3. The sequences were deposited in GenBank (www.ncbi.nlm.nih.gov/genbank/) with accession numbers for OXA-470 to OXA-479 being KR182163 to KR182172 respectively.

β-Lactamase production

The results for β-lactamase production for the 57 strains that were tested are shown in Table 1. Two of the 13 strains lacking genes for an OXA protein (15.4%) and 24 of the 44 with a gene for an OXA protein (54.5%) were positive in the cefinase nitrocefin disk test.

Antimicrobial susceptibility testing

The results of susceptibility testing also are summarized in Table 1. The MIC value for control strain 95/1000 was ≥128 µg ml⁻¹, consistent with a previous report (Mortimer-Jones et al., 2008). Four (21.9%) of the 13 strains lacking blaOXA-63 group genes were recorded as being resistant to ampicillin (21.9%), whilst 27 (61.4%) of the 44 where the gene was identified were resistant.
Comparison of presence of OXA protein, β-lactamase activity and ampicillin susceptibility

Of the 13 strains predicted to lack OXA proteins that were tested, four were resistant to ampicillin and two of these were disk negative (Table 1). The other nine strains were disk negative and susceptible to ampicillin.

Of the 44 strains with a predicted OXA protein, changes in the amino acid sequences outside the five active sites, as reflected in the different OXA designations, were not consistently associated with differences in β-lactamase activity and/or susceptibility to ampicillin. Twenty-four (54.5%) of these strains had a predicted OXA protein, showed β-lactamase production and were resistant to ampicillin. Seventeen strains (38.6%) were predicted to have a protein, but were β-lactamase negative and susceptible to ampicillin. These included three strains with OXA-136, two with OXA-137, eight with OXA-479 and one each of OXA-470, OXA-473, OXA-474 and OXA-475. Three strains (6.8%), all with OXA-136, lacked measurable β-lactamase activity but had low-level resistance to ampicillin.

Discussion

The purpose of this study was to investigate the distribution of \textit{bla}_{OXA-63} group genes in strains of \textit{B. pilosicoli} from different sources. Evidence for the presence of these genes was found in isolates from diverse geographical regions and host species, but these and other \textit{bla}_{OXA} genes were not found in other \textit{Brachyspira} species that were examined. These findings support the hypothesis that the \textit{bla}_{OXA-63} group genes were acquired ancestrally in \textit{B. pilosicoli} after it divided from the other evolving \textit{Brachyspira} species, and now represent resident genes specific to this species. The genes themselves are under evolutionary pressure, including that resulting from the inherent recombinant population structure of \textit{B. pilosicoli}. 
Consistent with the report of extensive genomic rearrangements being present in sequenced strains of *B. pilosicoli* (Mappley *et al.*, 2012), the position of the *bla*<sub>OXA-63</sub> group genes varied on the genomes in three of the four strains where the whole genome had been sequenced, and was absent from the genome of the fourth strain (P43/6/78<sup>T</sup>). Furthermore, these strains varied in the number of transposases identified in their genomes (from none to 34), and in the case of B2904 there were two transposases adjacent to the *Bla*<sub>OXA-63</sub> group gene. These transposases could be associated with rearrangement of the position of the gene on the genome, or even with lateral gene transfer to or from another strain or species, although there was no direct evidence for this. Indeed, as mentioned earlier, *bla*<sub>OXA-63</sub> group genes were not identified in the genomes of 32 strains of eight other *Brachyspira* species that were examined.

Sixteen *B. pilosicoli* strains apparently completely lacked a *bla*<sub>OXA-63</sub> group gene, including the type strain P43/6/78<sup>T</sup> where the whole genome sequence was available. Interestingly both the genes and conserved sequences around both ends of the gene were absent in all these strains, despite their diverse origins and different STs in MLST. It remains unclear whether this region containing the resident *bla*<sub>OXA-63</sub> group gene has been lost from these strains or if it was acquired by ancestors of the much larger subset of strains that still have the gene. Nine of the strains without the genes were from human beings and pigs in the Asaro valley in the Eastern Highlands of PNG, and it seems possible that in this unique and remote location strains may have existed that never had the genes or acquired them. On the other hand these strains were not all closely related, and furthermore there are no obvious epidemiological links between them and the other seven genetically distinct strains that lacked the genes, which were isolated from pigs, chickens and horses from Canada, the UK and from different parts of Australia. If the genes have been lost from all these strains the precise nature of the excision around the gene suggests that there is something about this region facilitating this loss. Examination of the
sequence around the site where the gene is missing in strain P43/6/78T unfortunately did not provide any further clues as to what might facilitate this putative excision.

Overall the bla\textsubscript{OXA-63} group genes or components of them were widely distributed amongst most \textit{B. pilosicoli} strains examined. The isolates were mainly recovered in the 1990s, and it would be useful to compare the results with those for more contemporary isolates. Only 44 of the 118 strains amplified with all three primer sets, but the other 58 strains amplifying with both or one or other of the internal primers, providing evidence for the presence of components of the genes. Amongst the latter 58 there were cases where strains isolated from the same species in the same location differed in their PCR profiles (eg STs 72 and 73; STs 117 and 118: table 1). In these cases a lack of PCR product may have resulted from minor changes in the primer binding sites, or have been associated with larger-scale gene rearrangements or deletions. These possibilities could not be determined without more extensive genomic sequencing of the strains. This subset of strains from which the genes were not sequenced represent additional diversity in the genomic region that was not captured in analysis of the genes in the 44 strains where they were sequenced.

OXA-63 has been reported to have a narrow spectrum of activity (Meziane-Cherif \textit{et al}., 2008). In this study ampicillin was used as a substrate since all class D \(\beta\)-lactamases significantly hydrolyse amino-penicillins (Poirel \textit{et al}., 2010). As might be predicted, most strains that lacked the OXA-63 group proteins did not show \(\beta\)-lactamases activity and were susceptible to ampicillin, whilst most of those with OXA-63 group proteins had \(\beta\)-lactamase activity and were resistant to ampicillin. Nevertheless this was not always the case. Two of the 13 strains tested that lacked \(\text{bla}_{\text{OXA-63}}\) group genes showed evidence of \(\beta\)-lactamase activity and were resistant to ampicillin, and in these cases it is likely that the ampicillin was hydrolysed by another distinct \(\beta\)-lactamase that has not previously been described in \textit{B. pilosicoli}. Two other
of the 13 strains had no β-lactamase activity detected and showed resistance to ampicillin, albeit at a low level. In these two strains ampicillin may have become less effective due to changes in the structure of the penicillin-binding proteins in the cell wall (Dassanayake et al., 2005), or for other reasons. Of the strains with OXA-63 group proteins, 39% did not have β-lactamase activity and were susceptible to ampicillin. Given the frequency of genetic recombination in this species, it seems likely that these \( \text{bla}_{\text{OXA-63}} \) group genes were not expressed as a result of being relocated to a genetic environment that did not promote expression. This apparent lack of expression was found with representatives of most of the OXA proteins. Three other isolates with \( \text{bla}_{\text{OXA-136}} \) group genes but no measurable β-lactamase activity had low-level resistance to ampicillin. This could have been due to low levels of expression of the enzyme and/or changes in the affinity of their penicillin binding proteins for ampicillin. In all these cases the exact reasons for inconsistent results requires additional investigation.

Conclusions

In this study genes encoding 10 previously un-described OXA proteins were identified in \( B. \) \( \text{pilosicoli} \). In most cases their presence in a strain was associated with β-lactamase activity and resistance to ampicillin. Where strains with the genes were susceptible this is likely to have been because the genes were in a genetic environment that that did not promoted their expression, probably as a consequence of the known frequent recombination and rearrangement events occurring in this spirochaete species. Despite evidence for these genes being fragmented and disrupted in many isolates, where they were present the \( \text{bla}_{\text{OXA-63}} \) group genes were relatively conserved. This suggests that selective pressure is exerted to maintain functional resistance genes, presumably driven by therapeutic use of penicillins. Penicillin has been used to treat infections with \( B. \) \( \text{pilosicoli} \) in humans (Kanavaki et al., 2002), but is also widely used to treat pigs and others species that may carry the spirochaete. A small subset of
isolates completely lacked $bla_{\text{OXA-63}}$ group genes, and it appeared that these might have been selectively excised from the genome at some point in the evolutionary history of the species. The mechanisms involved require further investigation.

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**References**


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**Figure legends**

**Fig 1.** Primer binding locations for the four PCR reactions designed to amplify blaOXA-63 group genes. Panel A: binding sites of primers Bp-oxa-U/D (external to the gene) and Bp-lac1-F192/R634 and Bp-oxa-F484/R692 (both internal to the gene). Panel B: binding site for P43-US/DS, targeted externally to the gene. A product size of 495 bp occurs in the absence of a \( \text{bla}_{\text{OXA-63}} \) group gene.

**Fig 2.** Nucleotide-based UPGMA tree showing the evolutionary relationships of the \( \text{bla}_{\text{OXA-63}} \) group genes in the tested strains including the four existing \( \text{B. pilosicoli} \) \( \text{bla}_{\text{OXA-63}} \) group sequences. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The graduations of the scale bar represents two nucleotide substitutions in 1000 base pairs of the gene fragment. Sequences for eight strains that had one of the four previously described \( \text{bla}_{\text{OXA-63}} \) group genes are shown in bold typeface. Genes from three of these were re-sequenced here (WesB, EU086830; COF-10, EU086832; Gap51.2, EU086833). The gene for Gap51.2, recorded as encoding OXA-136 and marked with an asterisk has been re-designated as \( \text{bla}_{\text{OXA-471}} \) encoding OXA-471.

**Fig 3.** Amino acid-based UPGMA tree showing the evolutionary relationships of the OXA-proteins in the 44 strains tested, as well as in five other strains recorded in GenBank. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The graduations of the scale bar represents five amino acid substitutions in 1000 base pairs of the sequenced gene fragment. The names of the four OXA proteins (OXA-63; OXA-136; OXA-137; OXA-192) previously described in \( \text{B. pilosicoli} \) and the ten new OXA proteins (OXA-470 through OXA-479; marked in bold) are shown to the right of the tree.
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