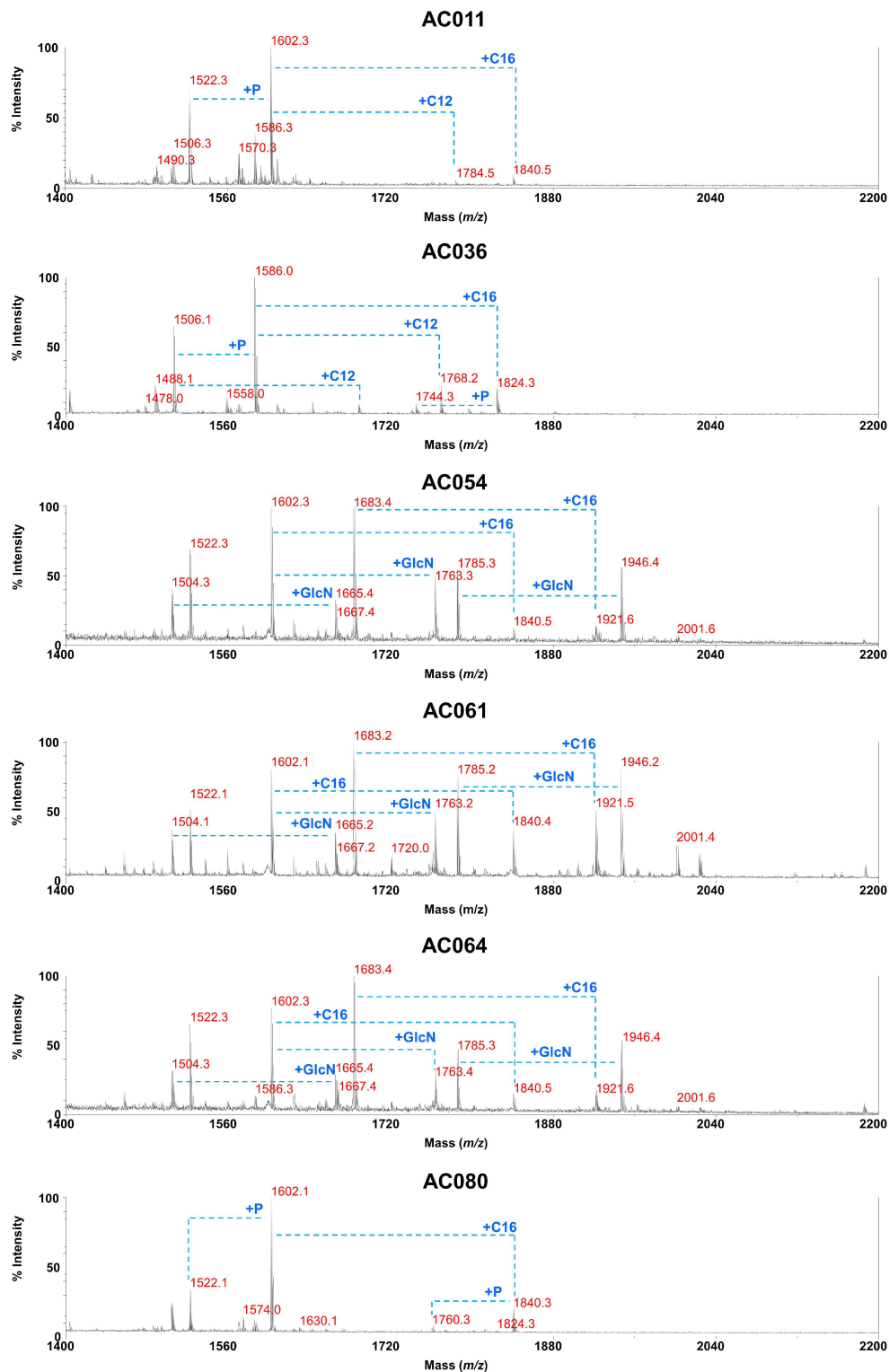


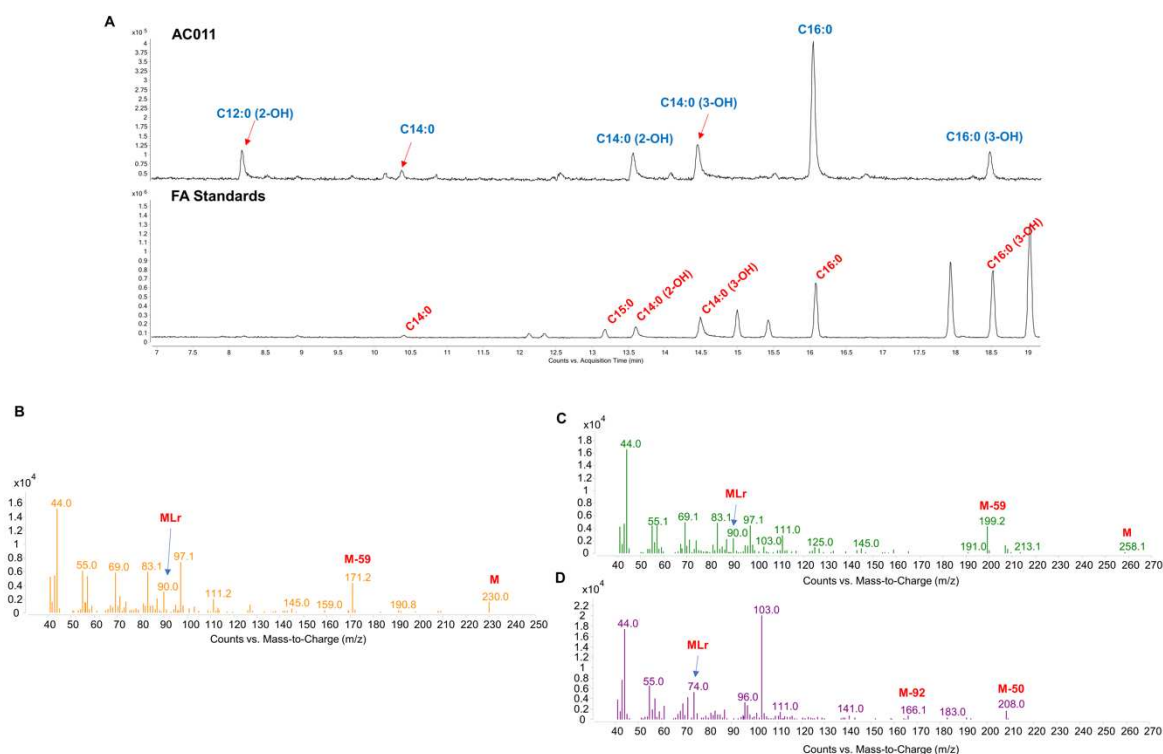
**Supplementary Figures S1-S3, TABLES S1 and S2, and Supplementary Datafile 1**

Polymyxin resistance and heteroresistance is common in clinical isolates of *Achromobacter* species and correlates with modifications of the lipid A moiety of the lipopolysaccharide

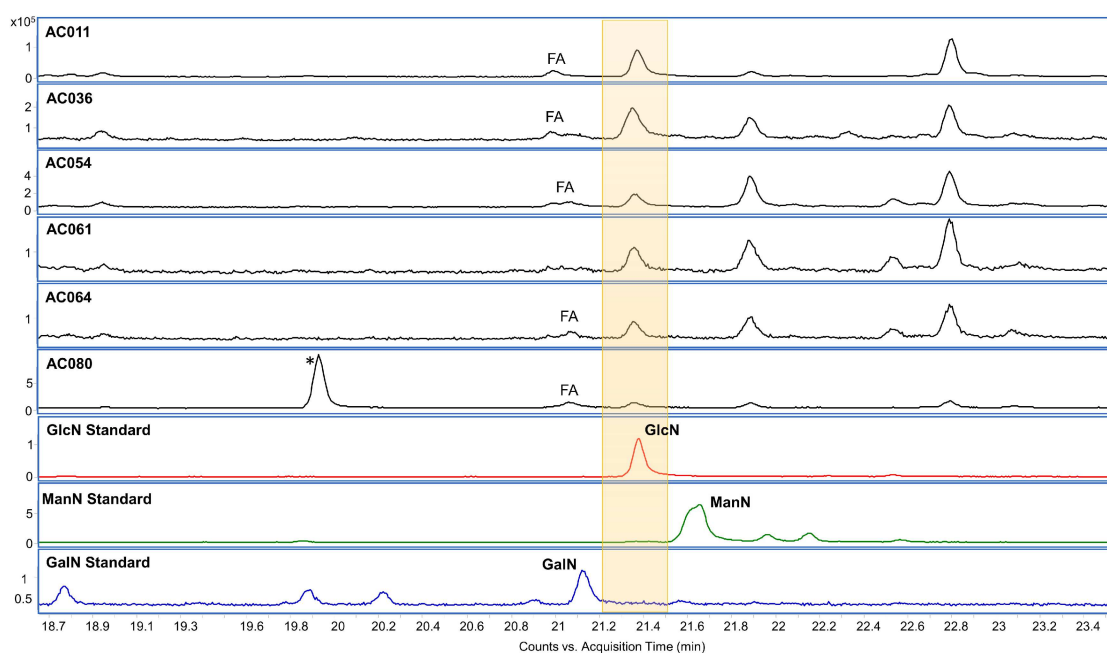
Lewis MacDonald, Sean Keenan, Flaviana Di Lorenzo, Nana E. Adade, Dervla T. D. Kenna, Beverley C. Millar, John E. Moore, José Ramos Vivas, Antonio Molinaro, and Miguel A. Valvano



**Figure S1.** Lipid A profile of *A. xylosoxidans* isolates AC011, AC036, AC061, AC064 and AC080, and *A. insuavis* AC054. Spectra were obtained via negative ion reflectron MALDI-TOF mass spectrometry using THAP as the matrix. Lipid A fractions were isolated as previously described (63). Ion peaks representing the gain of phosphate groups (+P), hydroxylaurate (+C12) and palmitate (+C16) acyl chains, and glucosamine residues (+GlcN) are indicated by blue dotted lines.



**Figure S2. A.** Zoom of the GC-MS chromatogram profile of the fatty acids as methyl ester derivatives of the lipid A from *A. xylosoxidans* isolate AC011 (top), chosen as a representative of the *Achromobacter* isolates analyzed in this study. The GC-MS chromatogram profile of opportunely prepared fatty acid methyl ester derivatives (bottom) is reported and compared with those obtained for *A. xylosoxidans* isolates AC011, AC036, AC061, AC064 and AC080, and *A. insuavis* AC054. **B, C,** and **D.** Electronic impact MS spectra that allowed the identification of C12:0(2-OH) (**B**), C14:0(2-OH) (**C**), and 14:0(3-OH) (**D**) in the lipid A preparations. “M” stands for molecular ion, while “MLr” indicates a fragment from McLafferty rearrangement. The detection of MLr ion at 90 u was diagnostic for the hydroxylation at C-2. On the contrary, the predominant ion at 103 u was indicative of the hydroxylation at C-3 as it is originated by the C $\beta$ –C $\gamma$  fission and it comprises the first three carbon atoms of the acyl chain and the positive charge is stabilized from the  $\beta$ -hydroxyl function.



**Figure S3.** Zoom of the GC-MS chromatogram profiles of the acetylated methyl glycoside derivatives of the HexN isolated from the lipid A preparations of *A. xylosoxidans* isolates AC011, AC036, AC061, AC064 and AC080, and *A. insuavis* AC054. The GC-MS chromatogram profiles of the acetylated methyl glycoside of glucosamine, mannosamine, and galactosamine opportunely prepared from authentical standards were used as a reference and reported in the figure. The comparison of the retention times clearly demonstrated that the sole HexN present in the lipid A of *Achromobacter* isolates was a GlcN. The yellow box indicates the peaks relative to the acetylated methyl glycoside of hexosamine, whereas the asterisk indicates a contaminant. “FA” stands for fatty acid.

**Table S1. Lipid A composition of *Achromobacter* species deduced from the observed  $m/z$  ion peaks**

$m/z$	Predicted lipid A structure <sup>a</sup>
1490	Penta-acyl, 4x 14:0, 1x C12:0, 1P
1506	Penta-acyl, 3x C14:0(OH), 1x C14:0, 1x C12:0(OH), 1P
1522	Penta-acyl, 3x C14:0(OH), 1x C12:0(OH), 1x C14:0, 1P
1559	Penta-acyl, 3x C14:0(OH), 1x 10:0(OH), 1x C14:0, 2P
1570	Penta-acyl, 3x C14:0(OH), 1x C14:0, 1x C12:0, 2P
1586	Penta-acyl, 3x C14:0(OH), 1x C14:0, 1x C12:0(OH), 2P
1602	Penta-acyl, 4x C14:0(OH), 1x C12:0(OH), 2P
1642	Penta-acyl, 4x C14:0(OH), 1x C16:0, 2P
1651	Penta-acyl, 3x C14:0(OH), 1x C14:0, 1x C12:0, 1P, GlcN
<b>1667*</b>	Penta-acyl, 3x C14:0(OH), 1x C14:0, 1x C12:0(OH), 1P, GlcN
1683	Penta-acyl, 4x C14:0(OH), 1x C12:0(OH), 1P, GlcN
1688	Hexa-acyl, 3x C14:0(OH), 1x C12:0(OH), 1x C14:0, 1x C12:0, 1P
1744	Hexa-acyl, 3xC14:0(OH), 1x C14:0, 1x C12:0(OH), 1x C16:0, 1P
1760	Penta-acyl, 4xC14:0(OH), 1x C12:0(OH), 1x C16:0, 1P
1768	Hexa-acyl, 4x C14:0(OH), 2x C12:0, 2P <b>or</b> 3x C14:0(OH), 1x C12:0(OH), 1x C14:0,
1x	C12:0, 2P
1785	Hexa-acyl, 4x C14:0(OH), 1x C10:0(OH), 1x 14:0, 2P
<b>1824*</b>	Hexa-acyl, 3x C14:0(OH), 1x C14:0, 1x C12:0(OH), 1x C16:0, 2P
1840	Penta-acyl, 4xC14:0(OH), 1x C12:0(OH), 1x C16:0, 2P
1889	Hexa-acyl, 3xC14:0(OH), 1x C16:0, 1x C12:0, 1x C12:0(OH), 1P, GlcN
1905	Hexa-acyl, 3xC14:0(OH), 1x C16:0, 1x C14:0, 1x C12:0(OH), 1P, GlcN
1921	Hexa-acyl, 4xC14:0(OH), 1x C16:0, 1x C12:0(OH), 1P, GlcN
1946	Hexa-acyl, 4x C14:0(OH), 1x C10:0(OH), 1x 14:0, 2P, GlcN
2001	Hexa-acyl, 4x C14:0(OH), 1x C12:0(OH), 1x 16:0, 2P, GlcN

\* The lipid A structures are predicted based on the observed  $m/z$  ion peaks. The canonical GlcN disaccharide is included in the calculations. GlcN, when indicated is the modification added to the phosphate residue. Peaks in bold with an asterisk may have alternative composition, which will require validation by more detailed structural analysis.

**Table S2.** Fatty acid content of lipid A isolated from *A. xylosoxidans* isolates AC011, AC036, AC061, AC064 and AC080, and *A. insuavis* AC054. A heterogenous composition was observed, which was however qualitatively equal for all the isolates. The absolute configuration of 2 and 3-hydroxy fatty acids remains to be defined.

<b>Fatty Acid Component</b>
12:0
14:0
16:0
10:0(3-OH)
12:0(2-OH)
12:0(3-OH)
14:0(2-OH)
14:0(3-OH)
16:0(3-OH)

## Supplemental Dataset 1

### MIC determination and population analysis profiles of all *Achromobacter* strains investigated in this study

MIC values were determined by broth microdilution (BMD) performed in duplicate for PmB concentrations between 1-256 µg/mL. Inocula were prepared by diluting an overnight broth culture of each isolate in CA-MHB to reach a final inoculum of  $5 \times 10^5$  CFU/ml. One honeycomb 96-well plate (Oy Growth Curves Ab Ltd, Finland) was used for each isolate. Each well received 180 µL of CA-MHB before the last well received 180 µL PmB at 569 µg/mL. The final well was then mixed via pipetting, the tip changed, and 180 µL transferred into the next well. This serial dilution was continued until 180 µL was discarded from the second to last well. Each well was then inoculated with 20 µL of the diluted bacteria. Plates were incubated at 37°C with continuous shaking in a Bioscreen C (Oy Growth Curves Ab Ltd, Finland) for 24 h taking automatic OD<sub>600</sub> readings every 30 min. The MIC endpoint was read as the lowest concentration of antibiotic at which the percent OD<sub>600</sub> relative to the no antibiotic control was ≤10%, which corresponded to no visible growth.

Population analysis was performed by calculating the percent of bacterial growth relative to the no PmB control from the OD<sub>600</sub> readings at 24 h in all PmB concentrations. Isolates were defined as heteroresistant when the antibiotic concentration exhibiting the highest inhibitory effects was ≥ 8-fold higher than the highest non-inhibitory concentration.

