

1 **Proteomic identification of Axc, a novel beta-lactamase with carbapenemase**
2 **activity in a meropenem-resistant clinical isolate of *Achromobacter***
3 ***xylosoxidans***

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26 Running title: *A. xylosoxidans* carbapenem resistance

27 **Abstract**

28

29 The development of antibiotic resistance during treatment is a threat to patients and their
30 environment. Insight in the mechanisms of resistance development is important for
31 appropriate therapy and infection control. Here, we describe how through the application of
32 mass spectrometry-based proteomics, a novel beta-lactamase Axc was identified as an
33 indicator of acquired carbapenem resistance in a clinical isolate of *Achromobacter*
34 *xylosoxidans*.

35 Comparative proteomic analysis of consecutively collected susceptible and a resistant
36 isolates from the same patient revealed that high Axc protein levels were only observed in
37 the resistant isolate. Heterologous expression of Axc in *Escherichia coli* significantly
38 increased the resistance towards carbapenems. Importantly, direct Axc mediated hydrolysis
39 of imipenem was demonstrated using pH shift assays and ¹H-NMR, confirming Axc as a
40 legitimate carbapenemase. Whole genome sequencing revealed that the susceptible and
41 resistant isolates were remarkably similar.

42 Together these findings provide a molecular context for the fast development of
43 meropenem resistance in *A. xylosoxidans* during treatment and demonstrate the use of
44 mass spectrometric techniques in identifying novel resistance determinants.

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48 **Introduction**

49

50 Development and spread of antibiotic resistance by pathogenic microorganisms is an
51 increasing healthcare problem. Moreover, certain resistance determinants spread readily^{1,2},
52 while the introduction of novel antibiotics is lagging behind. Several clinically important
53 classes of antimicrobials such as the beta-lactams, target the bacterial cell wall³. Resistance
54 to beta-lactams can be mediated by beta-lactamases that are capable of hydrolysing the
55 beta-lactam ring. Following the initial introduction of penicillin, second and third generation
56 beta-lactams have been developed which, in turn, triggered the selection of beta-
57 lactamases with broader specificities. Carbapenem treatment is often used as a last resort,
58 since extended-spectrum beta-lactamases (cephalosporinases) are becoming more
59 prevalent in Gram-negative bacteria. The emergence and spread of carbapenemases such as
60 class A KPC⁴, a number of metallo beta-lactamases^{5,6} (class B: IMP, VIM, NDM) and class D
61 oxacillinases such as OXA-48⁷, in combination with other resistance mechanisms⁸, can
62 jeopardize carbapenem efficacy, leaving little or no treatment options for patients.

63 *Achromobacter xylosoxidans* is a rod shaped aerobic non-fermentative Gram-
64 negative bacterium. It is widespread in the environment and generally considered as an
65 opportunistic pathogen. Chronic infections with *A. xylosoxidans* are problematic in cystic
66 fibrosis patients^{9,10} but reported prevalence numbers vary greatly (3-30%)^{11,12}. Moreover,
67 bacteremia as a result of *A. xylosoxidans* can occur in immunocompromised patients¹³. *A.*
68 *xylosoxidans* is notorious for its intrinsic high level of resistance, especially towards
69 penicillins and cephalosporins¹⁴⁻¹⁶. In general, carbapenem resistance in *A. xylosoxidans* is
70 not widespread and as a result meropenem treatment is routinely applied, even in the case
71 of recurring infections^{17,18}. Though carbapenem resistance is observed, specifically for

72 meropenem¹⁹, there are few reports on the mechanism of carbapenemase resistance in *A.*
73 *xylosoxidans*. Notable exceptions are the plasmid-encoded carbapenemase IMP^{20,21} and the
74 chromosomally encoded class D beta-lactamase OXA-114¹⁶, that show low level
75 carbapenemase activity. A comparative genomic exploration of two *A. xylosoxidans* isolates
76 revealed many genes that could be involved in drug resistance, such as efflux pumps and β-
77 lactamases. However, most of these genes were conserved between carbapenem
78 susceptible and resistant strains, highlighting the difficulty in translating genomic data to the
79 observed resistant phenotypes²².

80 In this study, two clinical isolates of *A. xylosoxidans* from an immunocompromised
81 patient with pneumonia were investigated. The initially cultured isolate from the respiratory
82 tract was susceptible to meropenem and treatment was started accordingly. However, a
83 subsequent meropenem resistant isolate was obtained from a blood culture after treatment
84 failure. Since PCR analysis was negative for known carbapenemases, we performed a
85 proteomic analysis which revealed the novel beta-lactamase Axc as highly abundant in the
86 meropenem-resistant, but not in the susceptible isolate. Axc expression led to an increase of
87 minimal inhibitory concentrations for carbapenems when introduced in a susceptible
88 *Escherichia coli* strain and direct carbapenemase activity of Axc was demonstrated using *in*
89 *vitro* imipenem conversion assays. Interestingly, the resistant as well as the susceptible
90 clinical isolates are genetically almost identical, emphasizing the importance of mass
91 spectrometry as a technique to investigate carbapenem resistance in *A. xylosoxidans*.

93 Results

94 **Development of meropenem resistance in *Achromobacter xylosoxidans* during treatment**

95 A 65-year old patient, diagnosed with chronic lymphocytic leukemia in 1989, underwent a
96 non-myeloablative stem cell transplantation in July 2014. In August 2014, the patient
97 developed neutropenic fever and pneumonia due to an infection with *A. xylosoxidans*. The
98 initial antibiogram (Supplemental Table 1) revealed a multi-resistant character, as is
99 commonly found for *A. xylosoxidans*, but the isolate was susceptible to meropenem. Hence,
100 meropenem treatment was initiated (4 dd 1 gram intravenously). During treatment the
101 patient developed a pneumothorax and died from septic shock 4 days later. At this point the
102 patient had been treated with meropenem for six days, as well as vancomycin and
103 antifungal therapy with liposomal amphotericin B. An antibiogram of a blood culture from a
104 sample taken one day before the patient's death revealed a meropenem-resistant *A.*
105 *xylosoxidans* phenotype (Supplemental Table 1). Subsequently, pure cultures from the first
106 and second clinical isolate were prepared (AchroS and AchroR, respectively). In line with the
107 antibiogram analysis described above, Etests showed that both isolates were resistant to
108 imipenem (MIC > 32 mg/L for both) but differed in their susceptibility towards meropenem
109 (MIC of 0.0094 mg/L (first isolate, AchroS) and 2 mg/L (second isolate, AchroR),
110 respectively). An in-house multiplex PCR assay (based on a published PCR³⁴) failed to detect
111 common carbapenemases (KPC, IMP, VIM, NDM and OXA-48, data not shown), suggesting
112 that the change in resistance is not mediated by these enzymes. This finding prompted us to
113 perform a comparative proteomic analysis of the two isolates to attempt to identify the
114 meropenem resistance mechanism.

116

117 **Comparative proteomic analysis shows differential levels of the beta-lactamase Axc**

118 Protein extracts of the meropenem-susceptible and resistant *A. xylosoxidans* isolates
119 (AchroS and AchroR) were first analysed by SDS-PAGE. Since no major visual differences
120 were observed (Supplemental Figure 1A), all bands were excised and processed for a
121 bottom-up LC-MS/MS proteomic analysis. In total, 2290 unique proteins were identified, of
122 which 1517 proteins were common to both isolates, while 226 and 537 were only found in
123 the resistant and susceptible isolates, respectively. For a semi-quantitative analysis, the
124 spectra assigned to peptides belonging to a certain protein were counted and compared
125 between the two different isolates (Supplemental Figure 1B). Of the uniquely observed
126 proteins, most are proteins with low spectral counts (often single peptide identifications),
127 likely resulting from sampling bias of low abundant proteins. One protein was observed with
128 100 spectra in the resistant isolate (AchroR) but none in the susceptible isolate (AchroS).
129 This protein, hereafter called Axc (for *Achromobacter xylosoxidans* carbapenemase,
130 GenBank ID: MF767301), is a putative PenP class A beta-lactamase (COG2367/pfam13354).

131 To confirm that Axc is highly abundant in the resistant isolate in comparison to the
132 susceptible isolate, a second proteomic analysis was performed on whole cell protein
133 extracts that were digested in-solution and analysed without any prior fractionation.
134 Spectral count analysis resulted in a cumulative quantification of 1356 different proteins, of
135 which 987 were found in both isolates, but 202 and 167 were uniquely quantified in the
136 resistant and susceptible isolates, respectively. The spectral count plot reflects the high
137 similarity of the two clinical isolates, with the vast majority of the proteins distributed along
138 the diagonal (Figure 1A). In accordance with the data described above, Axc is the most
139 prominent outlier in the resistant clinical isolate. A number of Axc tryptic peptides (Figure

140 1B) is clearly visible in the LC-MS/MS analysis of the resistant but not the susceptible
141 isolates (Figure 1C). Of note, cells used for these analyses were grown in the absence of
142 meropenem, so the high level of Axc in the resistant isolate is independent of antibiotic
143 pressure.

144

145 **Axc is present in meropenem resistant and susceptible *A. xylosoxidans* isolates**

146 To investigate whether *A. xylosoxidans* acquired the *axc* gene in the course of the
147 treatment, we performed a PCR for the *axc* open reading frame on both the resistant and
148 susceptible isolates AchroS and AchroR. This analysis demonstrated that *axc* is present in
149 both clinical isolates (Supplemental Figure 2). Moreover, Sanger sequence analysis
150 demonstrated that the sequence of *axc* is identical in both isolates (our unpublished
151 observations), indicating that the observed meropenem resistance is not likely caused by an
152 alteration of protein function.

153 A database search revealed that the *axc* gene is not present in all *A. xylosoxidans*
154 strains (Figure 2A). Like in our clinical isolates, *axc* is present in the NH44784_1996 strain
155 (Genbank identifier NC_021285.1)³⁵ but not in the strains NCBR 15126/ATCC27061
156 (Genbank chromosome CP006958.1) and C54 (Refseq assembly GCF_000186185.1) for
157 instance. In those strains that contain *axc*, the gene is located next to a putative LysR-type
158 transcriptional regulator (Pfam 03466), hereafter *axcR* (for axc-associated regulator).
159 Additional PCR and Sanger sequencing experiments verified that the intergenic regions in
160 AchroS and AchroR are identical. However, they differ at two positions with the intergenic
161 region in strain NH44784_1996 (Supplemental Figure 2B).

162 Our results show a high degree of similarity between the meropenem resistant and
163 susceptible strain, raising the possibility that these two strains represent a clonal complex.
164 To further explore the relatedness of both isolates, whole genome sequencing (WGS)
165 analysis was performed. This showed that both patient isolates are highly similar, with only
166 one single-nucleotide polymorphism (SNP) within the gene encoding AxyZ³⁶. This SNP was
167 confirmed by PCR and subsequent Sanger sequencing, ruling out the possibility that this was
168 an artefact of the assembly procedure. Moreover, *axc* and its putative regulator *axcR* were
169 found to be located in the same genomic region as in NH44784_1996 strain (Figure 2A).
170 Overall, the genome sequences suggest that both isolates are clonal, and that the
171 meropenem resistance evolved within the same strain during the course of treatment.

172 To demonstrate how Axc is related to other class A beta-lactamases, we compared
173 the Axc amino acid sequence with the sequence of another 176 representatives of this
174 family using an alignment consensus based on a report by Walther-Rasmussen and Hoiby³⁷.
175 The resulting unrooted cladogram shows that Axc is most closely related to a class A beta-
176 lactamase of *Rhodoferax saidenbachensis* (WP_029709665, Figure 2B). Only a limited
177 number of class A beta-lactamases have activity towards carbapenems, but none of these
178 cluster with the Axc sequence (Figure 2B). Our data therefore suggest a novel function for
179 the PenP family of beta-lactamases (COG2367) to which Axc belongs.

180

181 **Functional characterisation of Axc**

182 To establish whether Axc indeed has activity towards carbapenems, Axc was expressed in a
183 heterologous host and hydrolysis of carbapenems was measured indirectly and directly.

184 The *E. coli* C43 strain, suitable for the expression of toxic proteins³⁰, is susceptible to
185 carbapenems. We generated a derivative of C43 that allows for IPTG-dependent expression
186 a plasmid-based copy of *axc* (*E. coli_Axc* (JC107)). Susceptibility testing for imipenem and
187 meropenem showed that the MICs for these carbapenems increased 8-fold, following
188 induction of *Axc* expression (Table 1). Though these levels were lower than for the positive
189 control (KPC) for our assay, they were specific for *Axc* as the expression of an unrelated
190 protein (PPEP-1³¹) did not lead to an increase in MIC values (Table 1). Thus, expression of
191 *Axc* is correlated to resistance towards carbapenems.

192 To directly demonstrate carbapenemase activity, hydrolysis of imipenem was
193 monitored *in vitro* through colorimetric assays (Figure 3A) and ¹H-NMR (Figure 3B).
194 Imipenem hydrolysis results in the formation of a carboxylic acid, and monitoring the
195 accompanying pH drop colorimetrically is a well-established method for the detection of
196 carbapenemase activity^{32,38}. Indeed, hydrolysis of imipenem was readily observed using KPC
197 cells (without IPTG). Consistent with our previous results, imipenem hydrolysis was
198 observed for *E. coli* cells harbouring the *Axc* expression plasmid (*E. coli_Axc*) grown in the
199 presence, but not in the absence, of IPTG. As before, these results were specific for *Axc*, as
200 induction of PPEP-1 expression (*E.coli_control*) did not result in imipenem hydrolysis (Figure
201 3A). In a parallel assay, ¹H-NMR was used to directly observe the opening (hydrolysis) of the
202 lactam ring in imipenem (Figure 3B). The chemical shifts of the peaks change as a result of
203 this hydrolysis, with the protons closest to the ring opening undergoing the largest change.
204 The H-6 proton of imipenem generates an adequately resolved multiplet at 3.42 ppm that
205 decreases in intensity upon hydrolysis. Concomitantly, the doublet generated by the
206 protons of the methyl group (H-9) move upfield, resulting in a decrease of the doublet at 1.3

207 ppm. After 10 minutes incubation of bacterial cells with imipenem, hydrolysis of imipenem
208 was observed with *E. coli*_Axc grown in the presence, but not in the absence, of IPTG.
209 Hydrolysis was also apparent for KPC, but not for *E. coli* cells expressing PPEP-1
210 (*E.coli_control*)), even after long incubation times (10h). Under these conditions, imipenem
211 hydrolysis was also observed for samples with *E. coli* cells harbouring the Axc expression
212 plasmid grown in absence of IPTG, due to leaky expression from the inducible promoter.

213 Taken together, our data establish that Axc has carbapenemase activity.

214

215 Discussion

216

217

218 In this paper we identified a new resistance mechanism that explained the difference in
219 meropenem susceptibility of two clinical isolates of *A. xylosoxidans* that were collected
220 within two weeks during treatment. Using a combination of comparative proteomic
221 analyses and functional assays, we have shown that the class A beta-lactamase Axc is highly
222 abundant in the meropenem resistant isolate in comparison to the susceptible isolate and
223 that Axc has carbapenemase activity.

224 Detection of carbapenemases from sequence data is challenging. First, carbapenemases
225 belong to different subgroups of beta-lactamases which have probably evolved by
226 convergent evolution. Although sequence identities are moderate, most of the class A
227 carbapenemases have a disulphide bridge between Cys-69 and Cys-238, but this is
228 dispensable for activity against carbapenems³⁹ and our data show that Axc does not contain
229 these residues. Thus, the presence of these cysteine residues is no guaranteed predictor for
230 carbapenemase activity. Next, some studies also revealed a mechanism where beta-lactam
231 trapping, without actual degradation, can be involved in resistance towards carbapenems
232 when levels are sufficiently high. In such cases, concomitant loss of porins is often observed
233^{40,41}. Thus, it is crucial to determine whether a beta-lactamase actually induces hydrolysis of
234 carbapenems. Our NMR and pH-shift analyses data clearly demonstrate Axc-mediated
235 opening of the beta-lactam ring in imipenem. Finally, many unexplained mechanisms of
236 carbapenem resistance remain. For instance, a recent study demonstrated plasmid derived
237 carbapenem resistance in *Klebsiella pneumonia* strains which could not be explained by the
238 most common carbapenemases found (KPC-type). Even though none of the plasmid-

239 encoded genes were obvious candidates for the observed resistance towards carbapenems,
240 several TEM-homologs were detected ⁴². Though the prediction of the activity of a certain
241 beta-lactamase against carbapenems is not straightforward studies such as the present one
242 highlight that mass spectrometry approaches can be used to gain insight in the mechanism
243 of action and role of specific proteins in the observed phenotypes.

244 We do not know whether the high level of Axc is the only mechanism conferring
245 meropenem resistance to our isolate of *A. xylosoxidans*. When expressed from an inducible
246 promoter, Axc confers moderate resistance to carbapenems to *E. coli*; MIC values compared
247 to the KPC strain suggest that Axc has a lower efficiency than KPC, but may also indicate
248 lower overall levels of expression. Differing efficiencies in carbapenemases are well
249 documented, to the point where the activity of a specific class, such as OXA-48, is difficult to
250 detect but of great clinical importance ⁴³. Full biochemical characterization of Axc, including
251 kinetic experiments, is subject to further study. Such experiments, in combination with
252 crystallography analysis, will provide more insight in the activity of Axc against different
253 beta-lactams and could resolve the structural characteristics of the binding pocket which
254 facilitates its activity towards carbapenems. We note, however, that two other changes in
255 the antibiogram between the meropenem susceptible and resistant isolates involve beta-
256 lactam antibiotics. Augmentin (amoxicillin/clavulanate) resistance changed from
257 intermediate (8 mg/L) to resistant (>32 mg/L), and piperacilline/tazobactam from
258 susceptible (<=4 mg/L) to intermediate resistance (8 mg/L). This suggests that Axc may have
259 a broad substrate specificity and is insensitive to inhibition by clavulanate and tazobactam.
260 Strikingly, both the meropenem-susceptible and meropenem-resistant isolates were
261 resistant to imipenem (MIC values higher than the maximum concentration tested (32

262 mg/L)). This indicates that, notwithstanding the activity of Axc towards imipenem as
263 presented here, imipenem resistance in the clinical isolates is not dependent on Axc.
264 Differences in the sensitivities towards different carbapenems results from the chemical
265 differences between the individual drugs ⁴⁴ and are often linked to the differential
266 permeability of the outer cell membrane ^{45,46}.

267 The regulatory mechanism leading to higher levels of Axc expression are unclear. Sequence
268 analyses showed that the meropenem susceptible and resistant *A. xylosoxidans* clinical
269 isolates are highly similar, with no differences in the *axc* promotor and coding sequence, nor
270 in its putative regulator *AxcR* and the *axc-axcR* intergenic region. The only SNP we identified
271 is located in the gene encoding *AxyZ*, the TetR-type repressor of the *axyXY-oprZ* operon ⁴⁷.
272 This leads to an amino acid substitution (V29G) in a region of *AxyZ* that is involved in DNA
273 binding in other members of TetR family ⁴⁸. *AxyX*, *AxyY* and *OprZ* form an efflux pump of the
274 resistance-nodulation division (RND) family and are predominantly found in aminoglycoside
275 resistant *Achromobacter* species ⁴⁹⁻⁵¹. A recent paper showed higher expression levels of
276 *axyY* in *A. xylosoxidans* strains containing *AxyZ_Gly29*, suggesting that this mutation leads to
277 reduced repression of *AxyZ* ⁵². Closer inspection of our proteomics data indicates that also
278 *AxyX* and *AxyY* are more abundant in the resistant isolate (spectral counts of 62 vs 21 for
279 *AxyX* and 10 vs 2 for *AxyY* in the results presented in Figure 1). However, the difference is
280 not as pronounced as found for *Axc* and more accurate quantitative proteomics
281 experiments have to be performed to validate these data.

282 Mutations in TetR-like repressors have been linked to differences in carbapenem resistance
283 ⁵³. For example, a 162 bp deletion in *axyZ* has been identified in certain carbapenem
284 resistant strains ⁵⁴. However, even though *AxyZ_Gly29* leads to higher expression of *axyY*,

285 resulting in higher MIC values for aminoglycosides, fluoroquinolones and tetracyclines, no
286 correlation between *axyY* expression and meropenem resistance was observed ⁵². In line
287 with this observation, a deletion of *axyY* in several *A. xylosoxidans* strains is reported to
288 result in only a modest increase in the susceptibility towards carbapenems ⁴⁷. Taken
289 together, it is likely that AxyXY-OprZ per se contributes little if any to the meropenem
290 resistance phenotype of our clinical isolate. Instead, our data show a critical role for Axc and
291 suggest that *axc* expression is regulated by AxyZ. If this is indeed the case, we postulate that
292 the increase in the meropenem MIC for the ACH-CF-911_{V29G} strain ⁵² is accompanied by
293 increased Axc levels.

294 From the clinical perspective, the development of resistance to meropenem within days
295 following meropenem treatment is remarkable. Previous longitudinal analyses of different
296 *A. xylosoxidans* isolates from one patient have revealed large phenotypic and genetic
297 differences, for example in the resistance towards different classes of antibiotics but they
298 were generally performed over longer time periods ^{35,53,54}. Such changes are believed to be
299 the result of adaptive evolution of the initial strain which infected the patient, but there is
300 also evidence that genetically different strains of *A. xylosoxidans* can co-exist within the
301 same chronically infected individual ⁵⁵. Though we cannot exclude a co-infection, it is likely
302 that the mutation in *axyZ* in our case occurred during treatment.

303 Finally, from a diagnostic point of view, the presence of Axc in both the sensitive and
304 resistant *A. xylosoxidans* isolates complicates straightforward detection by molecular
305 methods in the future and warrants detection based on protein abundance levels.
306 Moreover, in addition to the now well-established application of MALDI-ToF-MS for
307 bacterial species identification, more elaborate mass spectrometry-based platforms clearly

308 have potential for the detection of resistance and virulence proteins^{23,56}, as exemplified by
309 the identification of Axc in meropenem resistant *A. xylosoxidans*.

310 **Methods**

311

312 All procedures performed in studies involving human participants were in accordance with
313 the ethical standards of LUMC Medical Ethical Committee and with the 1964 Helsinki
314 Declaration and its later amendments or comparable ethical standards.

315 **Materials**

316 MilliQ water was obtained from a Q-Gard 2 system (Merck Millipore, Amsterdam the
317 Netherlands). Acetonitrile of LC-MS grade was obtained from Biosolve (Valkenswaard, the
318 Netherlands). Porcine trypsin was purchased from Promega (Madison, WI). If not indicated
319 otherwise, chemicals were from Sigma Aldrich (St Louis, MN, USA).

320 **Susceptibility profiling of clinical isolates**

321 The minimum inhibitory concentrations (MICs) for different antibiotic compounds on the
322 clinical isolates (Clinical IDs: M 14073954-7 (first isolate), M 14076260-2 (second isolate))
323 were initially determined using a Vitek-2 system (bioMérieux, Marcy-l'Étoile, France).
324 Several colonies of plate grown cultures were inoculated and suspended in 0.45 % sterile
325 physiological saline solution. Suspensions for testing had densities between of approx. 0.5
326 McFarland standards. The testing procedure was performed according to the
327 manufacturer's instructions.

328 Pure cultures of the susceptible and resistant isolates (AchroS and AchroR,
329 respectively (Table 2)) were subjected to further susceptibility testing and used for the
330 proteomic and genomic analyses. Etests (bioMérieux) were performed according to the
331 recommendations from EUCAST (http://www.eucast.org/clinical_breakpoints/). EUCAST
332 does not provide recommendations for the interpretation of MIC values or clinical
333 breakpoints for *Achromobacter xylosoxidans*. Therefore, scoring was performed using locally
334 developed protocols that are based on clinical breakpoints for other non-fermentative
335 Gram-negative rods.

336 **Mass spectrometry-based proteomics**

337 Cells were grown in BHI (Oxoid, Basingstoke, UK). Cells were collected by centrifugation
338 (4000g, 5 min) from 1 mL cell culture and washed with phosphate- buffered saline (PBS, pH
339 7.4). Pellets were stored at -80° C until further use.

340 Two different proteomics experiments were performed. For the first, cell extracts from *A.*
341 *xylosoxidans* strains (AchroS and AchroR, Table 2) were prepared in LDS (Lithium dodecyl
342 sulphate) sample buffer (Novex, Thermo Scientific) and put at 95 °C for 5 minutes for cell
343 lysis and protein extraction. Proteins were separated on Novex precast 4–12% Bis-Tris gels
344 (Thermo Scientific, Waltham, MA, USA) with MOPS (3-(N-morpholino)propanesulfonic acid)
345 running buffer (Thermo Scientific). After overnight staining using a Colloidal Blue Staining Kit
346 (Thermo Scientific), destained gel lanes were processed into 31 slices per lane. Gel pieces
347 were sequentially washed with 25 mM ammonium bicarbonate and acetonitrile. Reduction
348 and alkylation were performed with dithiothreitol (DTT, 10 mM, 30 minutes at 56 °C) and
349 iodoacetamide (IAA, 55 mM, 20 min at room temperature) respectively. Following several
350 washes with 25 mM ammonium bicarbonate and acetonitrile, bands were overnight

351 digested with trypsin (12.5 ng/μl in 25 mM NH₄HCO₃). Digest solutions were lyophilized and
352 reconstituted in 0.5% trifluoroacetic acid (TFA). Nano-LC separation was carried out using a
353 Ultimate 3000 RSLC nano System equipped with an Acclaim PepMap RSLC column (C18, 75
354 μm x 15 cm with 2 μm particles, Thermo Scientific) preceded by a 2 cm Acclaim PepMap100
355 guard column (Thermo Scientific). Peptide elution was performed by applying a mixture of
356 solvents A and B with solvent A being 0.1% formic acid (FA) in water and solvent B 0.1% FA
357 in 80 % acetonitrile (ACN). Peptides were eluted from the column with a multi-step gradient
358 from 5% to 55% solvent B in 55 minutes, at a constant flow rate of 300 nL min⁻¹. MS analysis
359 was performed employing a maXis Impact UHR-TOF-MS (Bruker Daltonics, Bremen,
360 Germany) in data dependent MS/MS mode in the *m/z* 150-2200 range. Ten ions were
361 selected at a time, based on relative abundance and subjected to collision-induced-
362 dissociation with helium as collision gas. A 1 minute dynamic exclusion window was applied
363 for precursor selection.

364 For the second proteomics approach, in-solution digests were prepared as described
365 previously ²³. In short, following cell disruption proteins were solubilized in 50%
366 trifluoroethanol (TFE). Subsequent reduction with DTT and alkylation with IAA were
367 performed prior to overnight tryptic digestion. Samples were lyophilized and reconstituted
368 in 0.5% TFA for injection. The nano-LC system and solvents were the same as in the above
369 experiment, but using an Acclaim PepMap RSLC column (C18, 75 μm x 50cm with 2 μm
370 particles, Thermo Scientific) with a 2 cm Acclaim PepMap100 guard column (Thermo
371 Scientific). Peptides were eluted from the column with a multi-step gradient from 5% to 55%
372 of solvent B in 180 minutes, at a constant flow rate of 300 nL min⁻¹. MS analysis was carried
373 out as described above.

374

375 **Mass spectrometry data export and spectral count analysis**

376 Conversion of Bruker Impact files into mzXML format using CompassXport version 3.0.9, led
377 to an initial total of 1,207,329 MS/MS and 444,565 MS/MS spectra, for the gel and total
378 lysate based comparisons, respectively (in-solution total lysate digests were analysed twice
379 and the data were merged). These spectra were searched using a concatenated forward and
380 decoy strategy. The forward database was constructed from the 6386 unreviewed
381 sequences from Uniprot for the organism *A. xylosoxidans* NH44784-1996 (November 2015)
382 together with the cRAP contaminant sequences, as downloaded in January 2015. An in-
383 house developed program, Decoy version V1.0.1-2-gfddc, that preserves homology, amino-
384 acid frequency and peptide length distribution, was used with default flags to construct the
385 decoy search space, which was concatenated to the forward sequences. The search against
386 the resulting database was performed using Comet version 2014.02 rev.2, with precursor
387 mass tolerance equal to 50 ppm and a fragment bin width of 0.05 Da, considering only fully
388 tryptic digests with at most 2 missed cleavages. All cysteines were assumed
389 carbamidomethylated, while methionine oxidation and N-terminal acetylation were
390 regarded as variable modifications. The confidence of these results was assessed by means
391 of Xinteract from the Trans Proteomics Pipeline suite version 4.8.0, retaining peptides
392 longer than 6 amino acids and running in semi-parametric mode. A second in-house
393 developed program, Pepxmltool version 2.5.1, was used to construct a protein
394 quantification table based on spectral counting of only non-degenerate peptides (peptides
395 mapping to a single protein) with corresponding q-value of at most 1%. Plots of the resistant
396 versus susceptible quantifications of all proteins revealed for both methods a predominantly

397 linear relationship, suggesting the applicability of an in-house developed program, Qntdiff
398 version 0.1.1, to assign p-values to deviations from linear behaviour and provide lists of the
399 most significantly differential protein expression levels between the two isolates. Custom-
400 made Gnuplot scripts were written and run on Gnuplot version 4.6 patchlevel 4 to visualize
401 differential proteins.

402

403 **Bacterial culture and genomic DNA preparation**

404 AchromoS and AchromoR (Table 2) were cultured on trypticase soy agar plates (BioMérieux, Marcy
405 l'Etoile, France), inoculated into liquid medium brain-heart-infusion (BHI) broth (Oxoid,
406 Basingstoke, UK) and grown overnight (~16-hrs) at 37 °C. Cells were harvested, washed with
407 phosphate-buffered saline (PBS, pH 7.4), and genomic DNA extraction was performed using
408 a phenol-chloroform extraction as previously described ²⁴.

409 **Whole genome sequencing and SNP calling**

410 Paired-end multiplex libraries were created as previously described ²⁵. Sequencing was
411 performed on an Illumina Hiseq 2000 platform (Illumina, San Diego, Ca, USA), with a read-
412 length of 100 basepairs. High-throughput *de novo* assembly of sequenced genome was
413 performed as previously described ^{26,27}. The assemblies are then automatically annotated
414 using PROKKA ²⁸ with genus-specific databases from RefSeq ²⁹. To identify single nucleotide
415 polymorphisms (SNPs), the Illumina sequence data of the meropenem-susceptible *A.*
416 *xylosoxidans* isolate (AchromoS) was mapped on the assembled genome of the meropenem-
417 resistant isolate (AchromoR) using SMALT software (<http://smalt.sourceforge.net/>), after which
418 SNPs were determined as previously described ²⁵.

419

420 **PCR and heterologous expression of Axc**

421 To corroborate the proteomics results and confirm results from the whole genome
422 sequence analysis, the *axc* ORF, the *axc-axcR* intergenic region and part of the *axyZ* ORF
423 were Sanger sequenced at a commercial provider (Macrogen, Amsterdam, the Netherlands).
424 PCR products (for primers see Table 3) were sequenced using the same primers as those
425 used for generating the product from genomic DNA isolated from the *A. xylosoxidans*
426 isolates. We have submitted the Axc sequence to Genbank, ID MF767301.

427 To construct an *E. coli* strain expressing Axc with a C-terminal 6xHis-tag from an IPTG
428 (isopropyl β -D-1-thiogalactopyranoside) inducible promoter, the *axc* open reading frame
429 was amplified using primers Axfor2 and Axrev3 (Table 3), using Accuzyme polymerase (GC
430 Biotech, Alphen aan den Rijn, The Netherlands) and genomic DNA from *A. xylosoxidans*
431 (AchroR) as a template. The amplified PCR product was digested using NdeI (Bioké, Leiden,
432 The Netherlands) and Xhol (Roche, Almere, The Netherlands), and cloned into similarly
433 digested pET-21b(+), yielding plasmid pET21B/Axc. The *axc* expression region was confirmed
434 using Sanger sequencing. Expression of Axc-his6 was carried out in *E. coli* C43(DE3)³⁰ in
435 Luria-Bertani broth (Affymetrix, Cleveland, OH, USA) with ampicillin (50 μ g/mL) and 1mM
436 IPTG (GC Biotech, Alphen aan de Rijn, the Netherlands) for 3 hrs at 37 °C and verified by
437 immunoblotting using anti-His antibody (Agilent Technologies, Santa Clara, CA, USA).

438

439 **Susceptibility testing of *E. coli* expressing Axc**

440 Minimal inhibitory concentration (MIC) values for the carbapenems imipenem (Etest,
441 BioMérieux) and meropenem (microbroth dilution) were established for *E. coli* C43/pET21B-
442 Axc (strain JC107, Table 2) in the presence and absence of 1mM IPTG. For imipenem, cells
443 were grown overnight in LB broth at 37 °C in the presence of ampicillin (50 µg/mL). The
444 overnight cultures were diluted 1:100 in LB broth with ampicillin and grown to mid
445 logarithmic phase ($OD_{600nm} \sim 0.5$). Two hundred µl of bacterial culture was spread on LB-
446 ampicillin (50 µg/mL) plates (with or without 1 mM IPTG) and an Imipenem Etest
447 (BioMérieux) was applied. MIC values were determined after 24h incubation at 37°C. The
448 meropenem MIC values were established by microbroth dilution. Bacterial cultures in
449 logarithmic phase ($OD_{600nm} \sim 0.5$) were diluted into LB-ampicillin medium to an OD_{600nm} of
450 0.05 in the presence or absence of 1mM IPTG and subsequently seeded in a 96-well plate. A
451 two-fold serial dilution of meropenem (starting at 12.5 µg/mL) was made by adding equal
452 amounts of meropenem (25 µg/mL) to the first row, from which a two-fold dilution series
453 was made in the rest of the plate. Samples were investigated for growth by measuring the
454 OD_{600nm} after 24 hrs incubation at 37 °C, while shaking. The MIC was the lowest
455 concentration of meropenem at which no growth was observed. As controls for our assays,
456 a carbapenem resistant *Klebsiella pneumoniae* clinical isolate (KPC; JC113) and an unrelated
457 expression construct (JC108; which expresses PPEP-1³¹ in an IPTG-dependent manner in the
458 same *E. coli* C43 background, Table 2) were included.

459 **Colorimetric imipenem hydrolysis assay**

460 Overnight bacterial cultures were diluted 100 fold in LB-ampicillin (50 µg/mL) medium and
461 grown to exponential growth phase at 37 °C while shaking. At the time of induction, the
462 OD_{600nm} was determined, the cultures were split in two and 1mM IPTG was added to one of
463 the cultures, followed by a further incubation for 3 hrs at 37 °C while shaking. At T=3h, the

464 OD_{600nm} was determined and cells were harvested by centrifugation (4000 g, 5 min) and
465 stored at -20 °C overnight. Cells were resuspended in water to yield equal densities based
466 on measured OD_{600nm} values. Then, 7.5 µL of bacterial suspension was mixed with 25 µL of
467 imipenem/phenol red/ZnSO₄ solution (3 mg/mL imipenem, 0.35% (wt/vol) phenol red, pH
468 7.8, 70 µM ZnSO₄) and incubated at 37 °C for 1 hr. Conversion of imipenem leads to a pH
469 drop that can be visualized by the color change of the buffer from red to yellow ³². To
470 quantify this effect, the UV-Vis spectrum was determined with a Nanodrop ND1000
471 spectrophotometer (Thermo Scientific) and the ratio between the absorption peaks at 431
472 and 560 nm was taken as a measure of imipenem hydrolysis.

473 **NMR imaging of imipenem conversion**

474 All proton nuclear magnetic resonance (¹H-NMR) experiments were performed on a 600
475 MHz Bruker Avance II spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a
476 5-mm triple resonance inverse (TCI) cryogenic probe head with a Z-gradient system and
477 automatic tuning and matching. All experiments were recorded at 310 K. Temperature
478 calibration was done before each batch of measurements ³³. The duration of the π/2 pulses
479 was automatically calibrated for each individual sample using a homonuclear-gated nutation
480 experiment on the locked and shimmed samples after automatic tuning and matching of the
481 probe head. The samples were prepared by adding 70 µL imipenem aqueous solution (5
482 mg/mL) to 280 µL milliQ water. This solution was mixed with 350 µL 75 mM phosphate
483 buffer (pH 7.4) in water/deuterium oxide (80/20) containing 4.6 mM sodium 3-
484 [trimethylsilyl] d4-propionate. Twenty µL of bacterial cell suspension were added and the
485 sample was mixed. Samples were manually transferred into 5-mm SampleJet NMR tubes.
486 The cell suspension samples were kept at 6 °C on a SampleJet sample changer while queued

487 for acquisition. For water suppression, presaturation of the water resonance with an
488 effective field of $\gamma B_1 = 25$ Hz was applied during the relaxation delay. A 1D-version of the
489 NOESY (Nuclear Overhauser effect spectroscopy) experiment was performed with a
490 relaxation delay of 4 seconds. A NOESY mixing time of 10 ms was used during which the
491 water resonance was irradiated with the presaturation RF field. After applying 4 dummy
492 scans, a total of 98,304 data points covering a spectral width of 18,029 Hz were collected
493 using 16 scans. The Free Induction Decay was zero-filled to 131,072 complex data points,
494 and an exponential window function was applied with a line broadening factor of 0.3 Hz
495 before Fourier transformation. The spectra were automatically phased and baseline
496 corrected.

497 **Bioinformatic analysis**

498 Comparison of Axc with other class A beta lactamases was performed by multiple alignment
499 using the Geneious 9.0 (Biomatters Ltd, Auckland, New Zealand)) software algorithm for
500 Global alignment with free end gaps, cost Matrix Blosum62. The tree was then built using
501 Jukes-Cantor genetic distance model with the Neighbor Joining tree build method.

502 **Data availability**

503 Illumina raw reads were deposited at the European Nucleotide Archive (ENA). Study ID:
504 PRJEB19781. Sample IDs: ERS1575148 (AchroR) and ERS1575149 (AchroS).

505

506

507 **References**

508 1 van der Bij, A. K. & Pitout, J. D. The role of international travel in the worldwide spread of
509 multiresistant Enterobacteriaceae. *J Antimicrob Chemother* **67**, 2090-2100,
510 doi:10.1093/jac/dks214 (2012).

511 2 Marston, H. D., Dixon, D. M., Knisely, J. M., Palmore, T. N. & Fauci, A. S. Antimicrobial
512 Resistance. *Jama* **316**, 1193-1204, doi:10.1001/jama.2016.11764 (2016).

513 3 Jovetic, S., Zhu, Y., Marcone, G. L., Marinelli, F. & Tramper, J. beta-Lactam and glycopeptide
514 antibiotics: first and last line of defense? *Trends Biotechnol* **28**, 596-604,
515 doi:10.1016/j.tibtech.2010.09.004 (2010).

516 4 da Silva, R. M., Traebert, J. & Galato, D. Klebsiella pneumoniae carbapenemase (KPC)-
517 producing Klebsiella pneumoniae: a review of epidemiological and clinical aspects. *Expert
518 Opin Biol Ther* **12**, 663-671, doi:10.1517/14712598.2012.681369 (2012).

519 5 Hong, D. J. *et al.* Epidemiology and Characteristics of Metallo-beta-Lactamase-Producing
520 *Pseudomonas aeruginosa*. *Infect Chemother* **47**, 81-97, doi:10.3947/ic.2015.47.2.81 (2015).

521 6 Zmarlicka, M. T., Nailor, M. D. & Nicolau, D. P. Impact of the New Delhi metallo-beta-
522 lactamase on beta-lactam antibiotics. *Infect Drug Resist* **8**, 297-309, doi:10.2147/idr.s39186
523 (2015).

524 7 Poirel, L., Potron, A. & Nordmann, P. OXA-48-like carbapenemases: the phantom menace. *J
525 Antimicrob Chemother* **67**, 1597-1606, doi:10.1093/jac/dks121 (2012).

526 8 Findlay, J., Hamouda, A., Dancer, S. J. & Amyes, S. G. Rapid acquisition of decreased
527 carbapenem susceptibility in a strain of Klebsiella pneumoniae arising during meropenem
528 therapy. *Clin Microbiol Infect* **18**, 140-146, doi:10.1111/j.1469-0691.2011.03515.x (2012).

529 9 Hansen, C. R. *et al.* Inflammation in Achromobacter xylosoxidans infected cystic fibrosis
530 patients. *J Cyst Fibros* **9**, 51-58, doi:10.1016/j.jcf.2009.10.005 (2010).

531 10 De Baets, F., Schelstraete, P., Van Daele, S., Haerlynck, F. & Vaneechoutte, M.
532 Achromobacter xylosoxidans in cystic fibrosis: prevalence and clinical relevance. *J Cyst Fibros*
533 **6**, 75-78, doi:10.1016/j.jcf.2006.05.011 (2007).

534 11 LiPuma, J. J. The Changing Microbial Epidemiology in Cystic Fibrosis. *Clinical Microbiology
535 Reviews* **23**, 299-323, doi:10.1128/cmr.00068-09 (2010).

536 12 Parkins, M. D. & Floto, R. A. Emerging bacterial pathogens and changing concepts of
537 bacterial pathogenesis in cystic fibrosis. *Journal of Cystic Fibrosis* **14**, 293-304,
538 doi:10.1016/j.jcf.2015.03.012 (2015).

539 13 Gomez-Cerezo, J. *et al.* Achromobacter xylosoxidans bacteremia: a 10-year analysis of 54
540 cases. *Eur J Clin Microbiol Infect Dis* **22**, 360-363, doi:10.1007/s10096-003-0925-3 (2003).

541 14 Lambiase, A. *et al.* Achromobacter xylosoxidans respiratory tract infection in cystic fibrosis
542 patients. *Eur J Clin Microbiol Infect Dis* **30**, 973-980, doi:10.1007/s10096-011-1182-5 (2011).

543 15 Saiman, L. *et al.* Identification and antimicrobial susceptibility of Alcaligenes xylosoxidans
544 isolated from patients with cystic fibrosis. *J Clin Microbiol* **39**, 3942-3945,
545 doi:10.1128/jcm.39.11.3942-3945.2001 (2001).

546 16 Doi, Y., Poirel, L., Paterson, D. L. & Nordmann, P. Characterization of a naturally occurring
547 class D beta-lactamase from Achromobacter xylosoxidans. *Antimicrob Agents Chemother* **52**,
548 1952-1956, doi:10.1128/aac.01463-07 (2008).

549 17 Amoureaux, L. *et al.* Detection of Achromobacter xylosoxidans in Hospital, Domestic, and
550 Outdoor Environmental Samples and Comparison with Human Clinical Isolates. *Applied and
551 Environmental Microbiology* **79**, 7142-7149, doi:10.1128/aem.02293-13 (2013).

552 18 Nichols, K. R., Knoderer, C. A., Jackson, N. G., Manaloor, J. J. & Christenson, J. C. Success
553 With Extended-Infusion Meropenem After Recurrence of Baclofen Pump-Related
554 Achromobacter Xylosoxidans Meningitis in an Adolescent. *J Pharm Pract* **28**, 430-433,
555 doi:10.1177/0897190015585757 (2015).

556 19 Amoureaux, L. *et al.* Epidemiology and resistance of *Achromobacter xylosoxidans* from cystic
557 fibrosis patients in Dijon, Burgundy: first French data. *J Cyst Fibros* **12**, 170-176,
558 doi:10.1016/j.jcf.2012.08.005 (2013).

559 20 Chen, Z. *et al.* IMP-1 encoded by a novel Tn402-like class 1 integron in clinical
560 *Achromobacter xylosoxidans*, China. *Sci Rep* **4**, 7212, doi:10.1038/srep07212 (2014).

561 21 Yamamoto, M. *et al.* Molecular characterization of IMP-type metallo-beta-lactamases among
562 multidrug-resistant *Achromobacter xylosoxidans*. *J Antimicrob Chemother* **67**, 2110-2113,
563 doi:10.1093/jac/dks179 (2012).

564 22 Hu, Y. *et al.* Genomic insights into intrinsic and acquired drug resistance mechanisms in
565 *Achromobacter xylosoxidans*. *Antimicrobial agents and chemotherapy* **59**, 1152-1161,
566 doi:10.1128/aac.04260-14 (2015).

567 23 Fleurbaaij, F. *et al.* Capillary-electrophoresis mass spectrometry for the detection of
568 carbapenemases in (multi-)drug-resistant Gram-negative bacteria. *Anal Chem* **86**, 9154-
569 9161, doi:10.1021/ac502049p (2014).

570 24 Croucher, N. J. *et al.* Rapid pneumococcal evolution in response to clinical interventions.
571 *Science* **331**, 430-434, doi:10.1126/science.1198545 (2011).

572 25 Harris, S. R. *et al.* Evolution of MRSA during hospital transmission and intercontinental
573 spread. *Science* **327**, 469-474, doi:10.1126/science.1182395 (2010).

574 26 Knetsch, C. W. *et al.* Whole genome sequencing reveals potential spread of *Clostridium*
575 *difficile* between humans and farm animals in the Netherlands, 2002 to 2011. *Euro Surveill*
576 **19**, 20954 (2014).

577 27 Page, A. J. *et al.* Robust high-throughput prokaryote de novo assembly and improvement
578 pipeline for Illumina data. *Microb Genom* **2**, e000083, doi:10.1099/mgen.0.000083 (2016).

579 28 Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068-2069,
580 doi:10.1093/bioinformatics/btu153 (2014).

581 29 Pruitt, K. D., Tatusova, T., Brown, G. R. & Maglott, D. R. NCBI Reference Sequences (RefSeq):
582 current status, new features and genome annotation policy. *Nucleic Acids Res* **40**, D130-
583 D135, doi:10.1093/nar/gkr1079 (2012).

584 30 Miroux, B. & Walker, J. E. Over-production of proteins in *Escherichia coli*: mutant hosts that
585 allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol*
586 **260**, 289-298, doi:10.1006/jmbi.1996.0399 (1996).

587 31 Hensbergen, P. J. *et al.* *Clostridium difficile* secreted Pro-Pro endopeptidase PPEP-1
588 (ZMP1/CD2830) modulates adhesion through cleavage of the collagen binding protein
589 CD2831. *FEBS Lett* **589**, 3952-3958, doi:S0014-5793(15)00951-5
590 [pii];10.1016/j.febslet.2015.10.027 [doi] (2015).

591 32 Nordmann, P., Poirel, L. & Dortet, L. Rapid detection of carbapenemase-producing
592 Enterobacteriaceae. *Emerg Infect Dis* **18**, 1503-1507, doi:10.3201/eid1809.120355 (2012).

593 33 Findeisen, M., Brand, T. & Berger, S. A 1H-NMR thermometer suitable for cryoprobes. *Magn*
594 *Reson Chem* **45**, 175-178, doi:10.1002/mrc.1941 (2007).

595 34 van der Zee, A. *et al.* Multi-centre evaluation of real-time multiplex PCR for detection of
596 carbapenemase genes OXA-48, VIM, IMP, NDM and KPC. *BMC Infect Dis* **14**, 27,
597 doi:10.1186/1471-2334-14-27 (2014).

598 35 Jakobsen, T. H. *et al.* Complete genome sequence of the cystic fibrosis pathogen
599 *Achromobacter xylosoxidans* NH44784-1996 complies with important pathogenic
600 phenotypes. *PLoS One* **8**, e68484, doi:10.1371/journal.pone.0068484 (2013).

601 36 Bador, J., Amoureaux, L., Blanc, E. & Neuwirth, C. Innate aminoglycoside resistance of
602 *Achromobacter xylosoxidans* is due to AxyXY-OprZ, an RND-type multidrug efflux pump.
603 *Antimicrob Agents Chemother* **57**, 603-605, doi:10.1128/aac.01243-12 (2013).

604 37 Walther-Rasmussen, J. & Hoiby, N. Class A carbapenemases. *J Antimicrob Chemother* **60**,
605 470-482, doi:10.1093/jac/dkm226 (2007).

606 38 Tamma, P. D. *et al.* Comparison of 11 Phenotypic Assays for Accurate Detection of
607 Carbapenemase-Producing Enterobacteriaceae. *J Clin Microbiol* **55**, 1046-1055,
608 doi:10.1128/jcm.02338-16 (2017).

609 39 Smith, C. A. *et al.* Role of the Conserved Disulfide Bridge in Class A Carbapenemases. *J Biol
610 Chem* **291**, 22196-22206, doi:10.1074/jbc.M116.749648 (2016).

611 40 Goessens, W. H. *et al.* Antibiotic trapping by plasmid-encoded CMY-2 beta-lactamase
612 combined with reduced outer membrane permeability as a mechanism of carbapenem
613 resistance in *Escherichia coli*. *Antimicrob Agents Chemother* **57**, 3941-3949,
614 doi:10.1128/aac.02459-12 (2013).

615 41 van Boxtel, R., Wattel, A. A., Arenas, J., Goessens, W. H. & Tommassen, J. Acquisition of
616 Carbapenem Resistance by Plasmid-Encoded-AmpC-Expressing *Escherichia coli*. *Antimicrob
617 Agents Chemother* **61**, 01413-01416, doi:10.1128/aac.01413-16 (2017).

618 42 Cerqueira, G. C. *et al.* Multi-institute analysis of carbapenem resistance reveals remarkable
619 diversity, unexplained mechanisms, and limited clonal outbreaks. *Proc Natl Acad Sci U S A*
620 **114**, 1135-1140, doi:10.1073/pnas.1616248114 (2017).

621 43 Koroska, F. *et al.* Comparison of phenotypic tests and an immunochromatographic assay and
622 development of a new algorithm for OXA-48-like detection. *J Clin Microbiol* **55**, 877-883,
623 doi:10.1128/jcm.01929-16 (2016).

624 44 El-Gamal, M. I. *et al.* Recent updates of carbapenem antibiotics. *Eur J Med Chem* **131**, 185-
625 195, doi:10.1016/j.ejmech.2017.03.022 (2017).

626 45 Bornet, C. *et al.* Imipenem and expression of multidrug efflux pump in *Enterobacter
627 aerogenes*. *Biochem Biophys Res Commun* **301**, 985-990 (2003).

628 46 Yang, F. C., Yan, J. J., Hung, K. H. & Wu, J. J. Characterization of ertapenem-resistant
629 *Enterobacter cloacae* in a Taiwanese university hospital. *J Clin Microbiol* **50**, 223-226,
630 doi:10.1128/jcm.01263-11 (2012).

631 47 Bador, J., Amoureaux, L., Blanc, E. & Neuwirth, C. Innate aminoglycoside resistance of
632 *Achromobacter xylosoxidans* is due to AxyXY-OprZ, an RND-type multidrug efflux pump.
633 *Antimicrob Agents Chemother* **57**, 603-605, doi:10.1128/aac.01243-12 (2013).

634 48 Ramos, J. L. *et al.* The TetR family of transcriptional repressors. *Microbiol Mol Biol Rev* **69**,
635 326-356, doi:10.1128/mmbr.69.2.326-356.2005 (2005).

636 49 Anes, J., McCusker, M. P., Fanning, S. & Martins, M. The ins and outs of RND efflux pumps in
637 *Escherichia coli*. *Front Microbiol* **6**, 587, doi:10.3389/fmicb.2015.00587 (2015).

638 50 Bador, J. *et al.* First description of an RND-type multidrug efflux pump in *Achromobacter
639 xylosoxidans*, AxyABM. *Antimicrob Agents Chemother* **55**, 4912-4914,
640 doi:10.1128/aac.00341-11 (2011).

641 51 Bador, J. *et al.* Distribution of innate efflux-mediated aminoglycoside resistance among
642 different *Achromobacter* species. *New Microbes New Infect* **10**, 1-5,
643 doi:10.1016/j.nmni.2015.11.013 (2016).

644 52 Bador, J. *et al.* Role of AxyZ Transcriptional Regulator in Overproduction of AxyXY-OprZ
645 Multidrug Efflux System in *Achromobacter* Species Mutants Selected by Tobramycin.
646 *Antimicrob Agents Chemother* **61**, e00290-00217, doi:10.1128/aac.00290-17 (2017).

647 53 Ormerod, K. L., George, N. M., Fraser, J. A., Wainwright, C. & Hugenholz, P. Comparative
648 genomics of non-pseudomonad bacterial species colonising paediatric cystic fibrosis patients.
649 *PeerJ* **3**, e1223, doi:10.7717/peerj.1223 (2015).

650 54 Ridderberg, W., Nielsen, S. M. & Norskov-Lauritsen, N. Genetic Adaptation of
651 *Achromobacter* sp. during Persistence in the Lungs of Cystic Fibrosis Patients. *PLoS One* **10**,
652 e0136790, doi:10.1371/journal.pone.0136790 (2015).

653 55 Dupont, C., Jumas-Bilak, E., Michon, A. L., Chiron, R. & Marchandin, H. Impact of High
654 Diversity of *Achromobacter* Populations within Cystic Fibrosis Sputum Samples on
655 Antimicrobial Susceptibility Testing. *J Clin Microbiol* **55**, 206-215, doi:10.1128/jcm.01843-16
656 (2017).

657 56 Charretier, Y. *et al.* Rapid Bacterial Identification, Resistance, Virulence and Type Profiling
658 using Selected Reaction Monitoring Mass Spectrometry. *Sci Rep* **5**, 13944,
659 doi:10.1038/srep13944 (2015).

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665

666 Author contribution statement

667 F.F., H.C.v.L., S.T.N., E.J.K. and P.J.H. designed the research. F.F., J.C., C.W.K, I.D., N.K., A.V.,
668 H.C.v.L. and P.J.H. performed experiments. F.F., A.A.H., J.C., C.W.K, W.K.S., M.G., N.K., T.D.L..
669 A.V. and P.J.H. analyzed data. F.F., J.C., W.K.S., H.C.v.L., E.J.K. and P.J.H. wrote the paper

670

671 Competing interests: The authors declare that they have no competing interests

672

673 **Figure legends**

674

675 **Figure 1: Comparative proteomic analysis of meropenem resistant and susceptible *Achromobacter***
676 ***xylosoxidans* clinical isolates**

677 A: Tryptic digests of protein extracts of the meropenem resistant (AchroR) and susceptible (AchroS)
678 isolate were analysed by LC-MS/MS. Spectra were assigned to peptides based on database
679 searching. Identified spectra were then assigned to the corresponding proteins and the total number
680 of spectra per protein were counted. Each circle represents one protein with the number of spectra
681 observed in the resistant and the susceptible isolate. Hence, proteins on the diagonal were observed
682 in similar counts in both isolates. Axc (arrow), a classA PenP-family beta-lactamase, is the most
683 prominent outlier.

684 B: The full amino acid sequence of Axc, with the peptides identified by LC-MS/MS analysis
685 underlined. Conserved residues from serine beta-lactamases, Ser-X-X-Lys, Ser-Asp-Asn and the active
686 site Glu, are in bold (37).

687 C: Extracted ion chromatograms of *m/z* values corresponding to tryptic peptides of Axc in the
688 meropenem resistant isolate (AchroR, upper panel) and susceptible isolate (AchroS, lower panel).
689 The corresponding tryptic peptides are indicated above the corresponding peaks.

690

691

692 **Figure 2: Genomic context of *axc* in *Achromobacter xylosoxidans* strains and comparison of Axc**
693 **with other class A beta-lactamases**

694 A: Axc (a putative PenP class A beta-lactamase, 1.17 e⁻⁵⁴) and the gene encoding its putative
695 transcriptional repressor (*axcR*), were found in both clinical isolates (AchroS and AchroR). Three
696 other fully sequenced genomes of *Achromobacter xylosoxidans* were examined for the presence of
697 *axc*; NH44784_1996 (Genbank identifier NC_021285.1), C54 (Refseq assembly GCF_000186185.1)
698 and NBRC_15126/ATCC27061 (Genbank chromosome CP006958.1). Only within the strain
699 NH44784_1996 (used as a reference to search our proteomics data), *axc* and the putative regulator
700 are also present.

701

702 B: Unrooted cladogram obtained for 176 class A beta-lactamases including Axc. The class A β -
703 lactamase protein sequences of Gram negative bacteria were obtained by querying the
704 refseq_protein database using Blastp (<1e-10, <http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi>) and a
705 consensus β -lactamase-alignment (37). Duplicate sequences and sequences causing a strong
706 overrepresentation of branches produced in the tree were removed. Names of known
707 carbapenamases (orange) and names of identifier of branches (37) are indicated. Axc is indicated with
708 a red dot. The closest homologue to Axc, a β -lactamase from *R. saidenbachensis* (WP_029709665),
709 is also indicated.

710 **Figure 3: Axc has carbapenemase activity.**

711 A: Axc was expressed in *E. coli* and cell extracts were tested for the ability to hydrolyse imipenem.
712 Bars show the A431/A560 ratio, which is a measure for the shift in pH due to imipenem hydrolysis.

713 B: NMR-based identification of imipenem hydrolysis by Axc. The structure of imipenem is shown,
714 with the proton numbering used in the spectrum. The line color indicates the incubation time (red,
715 10 min at room temperature or black, 10 hrs at 6 °C). Imipenem hydrolysis is accompanied by the
716 loss of the H-6 multiplet at 3.4 ppm, and a shift in the H-9 doublet, resulting in a decrease of the
717 doublet at 1.3 ppm.

718 Strains used (see also Table 2): KPC (JC113): Carbapenem resistant *Klebsiella pneumoniae*. *E.coli* Axc
719 (JC107): *E. coli* strain C43(DE3), containing plasmid pET21-Axc; *E.coli*_control (JC108): *E. coli* strain
720 C43, containing plasmid pET21-PPEP-1 (Pro-Pro endopeptidase 1). IPTG: isopropyl β-D-1-
721 thiogalactopyranoside

722

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724

725 **Table 1: Effect of Axc expression in *E. coli* on the susceptibility towards imipenem and meropenem**

726 Minimal inhibitory concentrations (MICs) of imipenem and meropenem for *E.coli* CA43 expressing
727 Axc (*E. coli_Axc*), or PPEP-1 (*E. coli_control*) and *Klesbsiella pneumoniae* expressing KPC
728 carbapenemase (KPC). N.D: Not detectable. IPTG: isopropyl β-D-1-thiogalactopyranoside.

729

Strain	MIC (mg/L)			
	Imipenem		Meropenem	
	- IPTG	+ IPTG	-IPTG	+ IPTG
<i>E. coli_Axc</i> ^a	0,5	4	1,563	12,5
<i>E. coli_control</i> ^b	0,38	N.D.	0,391	0,391
KPC ^c	>32	>32	>12,5	>12,5

730

731 ^aJC107: *Escherichia coli* C43/pET21B-Axc

732 ^b JC108: *Escherichia coli* C43/pET21B/PPEP-1 (Pro-Pro endopeptidase 1)

733 ^c JC113: Carbapenemase-positive *Klebsiella pneumoniae*

734

735

736 **Table 2: Strains used in this study.** Axc: *Achromobacter xylosoxidans* carbapenemase. PPEP-1: Pro-
737 Pro endopeptidase 1 (31)

738

Name	Short description	Full description
JC188	AchroS	Meropenem susceptible <i>A. xylosoxidans</i> clinical isolate
JC186	AchroR	Meropenem resistant <i>A. xylosoxidans</i> clinical isolate
JC107	<i>E. coli</i> _Axc	<i>Escherichia coli</i> C43/pET21B-Axc
JC108	<i>E. coli</i> _control	<i>Escherichia coli</i> C43/pET21B-PPEP-1
JC113	KPC	Carbapenemase positive <i>Klebsiella pneumoniae</i>

739

740

741 **Table 3: Primers used in this study.** Axc: *Achromobacter xylosoxidans* carbapenemase. axcR: *axc*-
742 associated regulator. axyZ: TetR-type repressor of the *axyXY-oprZ* operon (36)

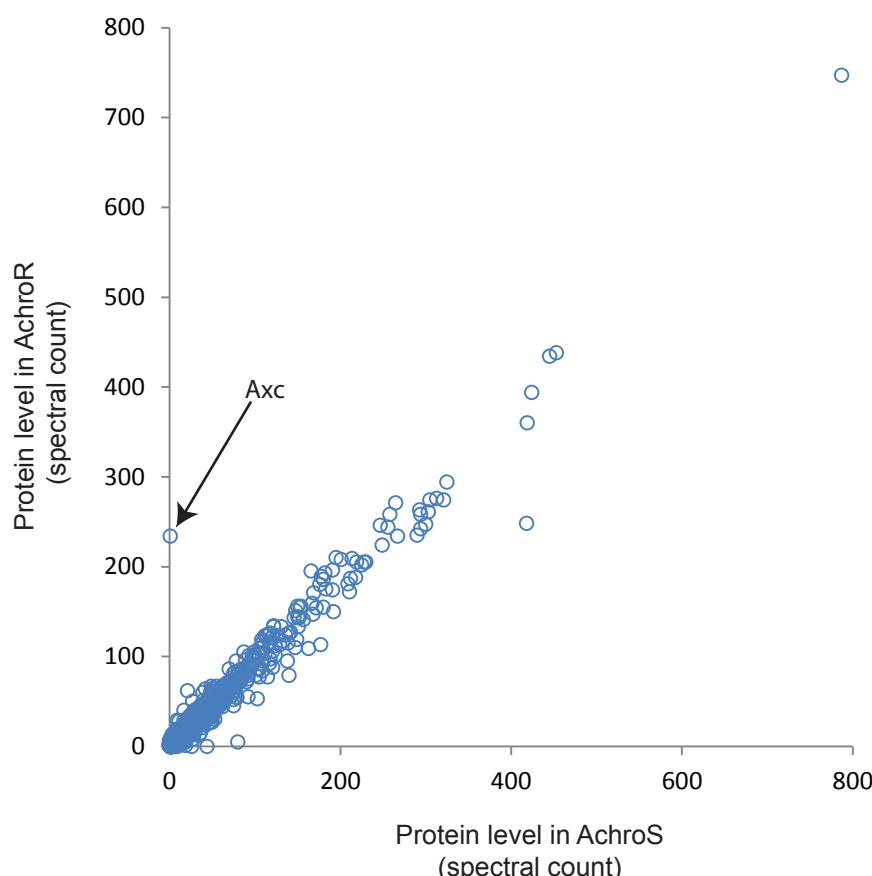
743

Name	5'> 3' sequence	Description
AxFor	5'-GAATGACATGTTGACCCGAAGAACCTTCATTGCC-3'	<i>axc</i> ORF forward
AxRev	5'-GCCGGATCCCTAGCCCAATGCCGCCACCAGCCTG-3'	<i>axc</i> ORF reverse
Operonfor	5'-CTGAGCATCAGGAAGCGTT-3'	<i>axc-axcR</i> intergenic region forward
Operonrev	5'-TCGAAGGATTGGACAAACAC-3'	<i>axc-axcR</i> intergenic region reverse
AC_RR_fw	5'-AGAAGAATCCCAACGCACCC-3'	Confirmation of SNP in <i>axyZ</i> , forward
AC_RR_rev	5'-TCGAGGCATACAGCGATTCC-3'	Confirmation of SNP in <i>axyZ</i> , reverse
Axfor2	5'-AAACATATGTTGACCCGAAGAACCTTCATTG-3'	Cloning <i>axc</i> ORF into pET21B forward
Axrev3	5'-AAACTCGAGGCCAATGCCGCCACCAGCC-3'	Cloning <i>axc</i> ORF into pET21B reverse

744

745

A



B

MLTRRTFIASAVLAGWIPALAHARTDKKTRWTRESLAAFQQGLAQVEAASRGRLGVALD
VGSGQAAGYRADERFLMLSSFKTLSAAYVLARADRGEDQLSRRIPITDADVREYSPVTRL
HVGPRGMTLAELEATITTSDNAAVNLMHKSYGGPQALTRYLRSLGDTVTRHDREPELN
RHPSEPDQDTTPQAMARTLDLFLFGDALKPQSQQQIQLQSWLLANTTGGKRLRAGMPADWK
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C

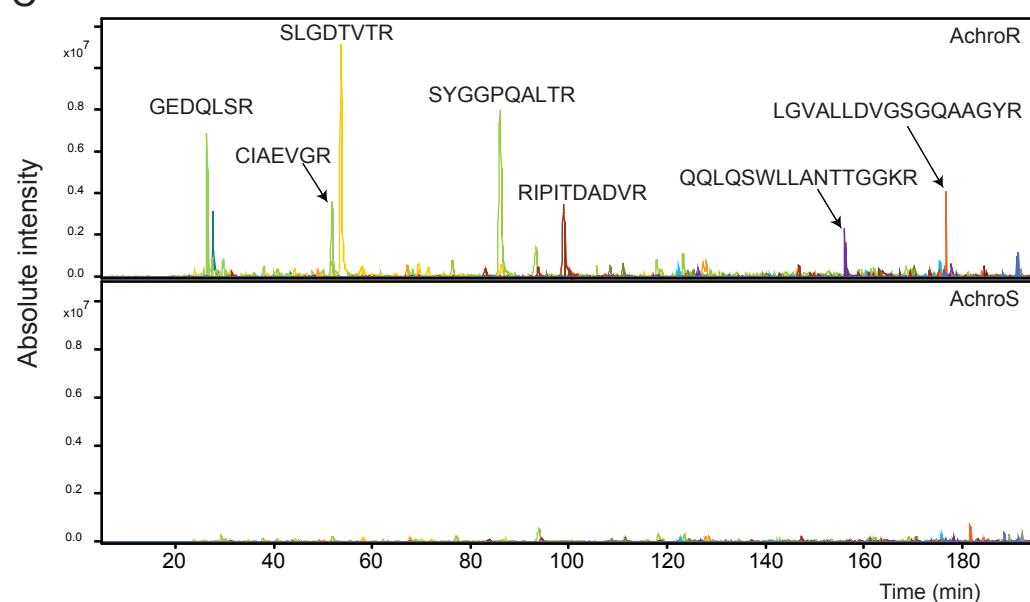
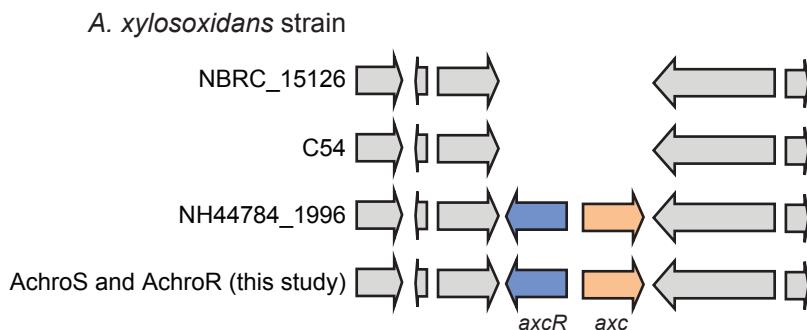


Figure 1

A



B

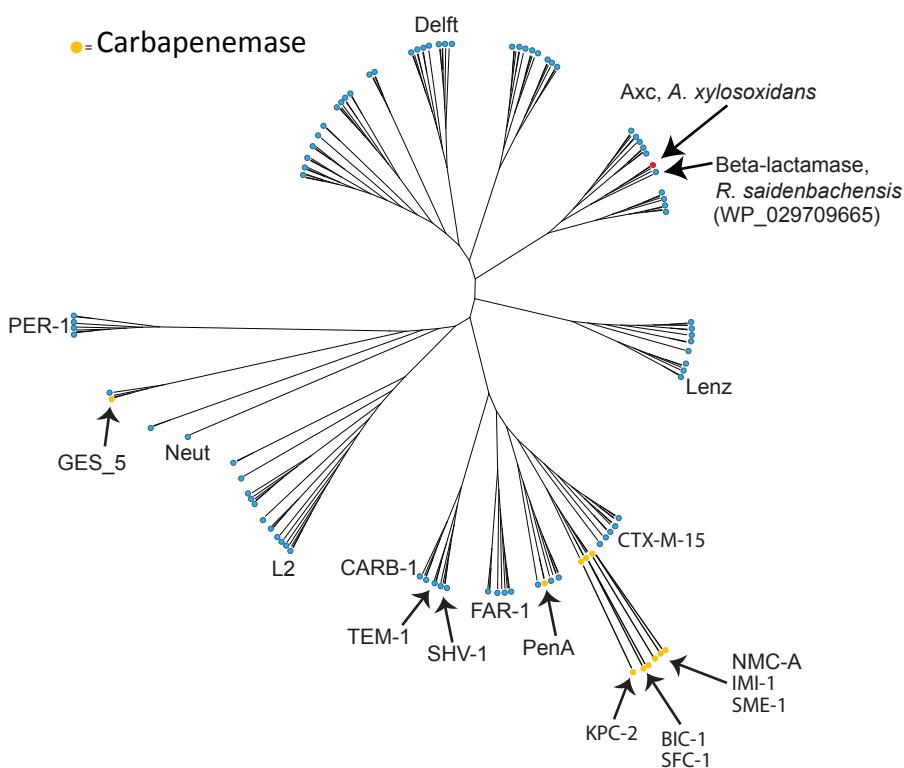
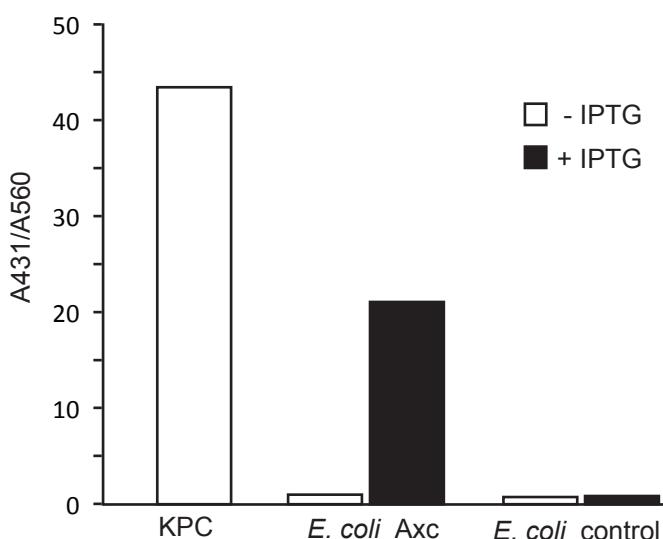


Figure 2

A



B

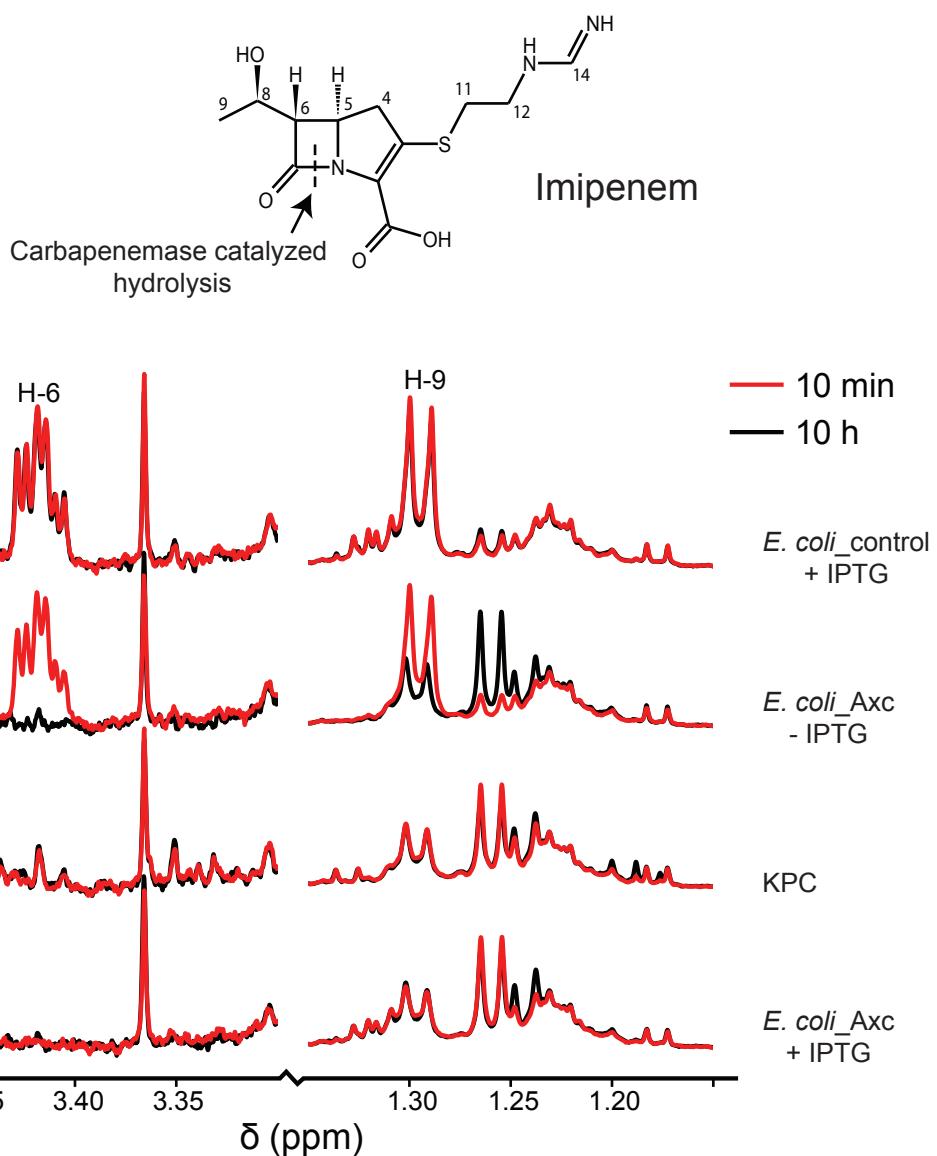


Figure 3