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1 **A compendium of antibiotic-induced transcription profiles reveals**
2 **broad regulation of *Pasteurella multocida* virulence genes**

3 E. Melnikow^a, C. Schoenfeld^b, V. Spehr^a, R. Warrass^a, N. Gunkel^a, M. Duszenko^b, P. M. Selzer^a, H. J.
4 Ullrich^{a*}

5 ^a Intervet Innovation GmbH, Zur Propstei, 55270 Schwabenheim, Germany

6 ^b Interfakultäres Institut für Biochemie Universität Tübingen, Hoppe-Seyler-Strasse 4, 72076 Tübingen,
7 Germany

8 *Corresponding author: phone: ++49-(0)6130-948267, fax: ++49-(0)6130-948517

9 joachim.ullrich@intervet.com

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10 Abstract

11 The transcriptional responses of *Pasteurella multocida* to eight antibiotics with known mode of actions
12 (MoAs) and one novel antibiotic compound with an unknown MoA were collected to create a
13 compendium of transcriptional profiles for MoA studies. At minimal inhibitory concentration the three
14 bactericidal compounds enrofloxacin, cefquinome and the novel compound had a minor impact on
15 gene regulation with approximately 1 % of the *P. multocida* genome affected, whilst the bacteriostatic
16 compounds florfenicol, tilmicosin, rifampin, trimethoprim and brodimoprim regulated 20 % of the
17 genome. Novobiocin was special in that it regulated 40 % of all *P. multocida* genes. Regulation of
18 target genes was observed for novobiocin, rifampin, florfenicol and tilmicosin and signature genes
19 were identified for most antibiotics. The transcriptional profile induced by the novel compound was
20 unrelated to the compendium profiles suggesting a new MoA. The transcription of many *P. multocida*
21 virulence factors, particularly genes involved in capsule synthesis and export, LPS synthesis,
22 competence, adherence and iron transport were altered in the presence of antibiotics. Virulence gene
23 transcription was mainly negatively affected, however the opposite effect was also observed in the
24 case of rifampin where the up-regulation of the *tad* locus involved in tight adherence was seen.
25 Novobiocin and trimethoprim caused a marked reduction in the transcription of capsule genes, which
26 correlated with a concomitant reduction of the capsular layer on the surface of *P. multocida*. The broad
27 negative impact on virulence gene transcription supports the notion that the therapeutic effect of some
28 antibiotics could be a combination of growth and virulence inhibition.

29
30 Keywords: *Pasteurella multocida*; Antibiotic; Microarray; Virulence

31
32 **1. Introduction**

33 Bacteria respond to antibiotic stress with a transcriptional reflex to counteract the assault on essential
34 processes such as cell wall synthesis, translation, transcription and replication. A very intuitive
35 response to an antibiotic attack is the up-regulation of target gene transcription to compensate the
36 inhibited target molecules. As the inhibition of target proteins causes alterations in the metabolic
37 network of the cell, antibiotic-induced gene regulation is not limited to target genes, but triggers a
38 complex secondary transcriptional response in an attempt to balance stressed physiology. The extent
39 of regulation in response to an antibiotic attack is time- and dose-dependent (Lin et al., 2005; Shaw et

40 al., 2003) and leads to an avalanche of regulatory responses often affecting the transcription of
41 hundreds of genes. Global analysis of transcriptional data showed that antibiotics can leave
42 transcriptional traces that reveal the inhibited target and often the transcriptional profile can be used
43 as a characteristic transcriptional fingerprint for a particular antibiotic, substance class or inhibited
44 target. Examples are ribosomal inhibitors like macrolides, which induce the transcription of ribosomal
45 genes (Ng et al., 2003), or the fluoroquinolones, which induce the transcription of SOS response
46 genes (Gmuender et al., 2001; Kaldalu et al., 2004). When the transcriptional profiles of antibiotics
47 with known MoAs are compiled into a database, a compendium of transcriptional profiles is created,
48 which allows the comparison with transcriptional responses of novel compounds with unknown MoAs.
49 A match in profiles suggests that the novel compound inhibits a target also exploited by a
50 compendium antibiotic, while a distinct profile would therefore point to a new MoA. Compendiums of
51 transcriptional profiles from gram positive and gram negative bacteria have increasingly been used to
52 obtain first indications of the MoAs of novel compounds (Boshoff et al., 2004; Hutter et al., 2004;
53 Freiberg et al., 2005). However, these studies differed with respect to the organisms, the antibiotics,
54 the dose and the duration of treatment, making it difficult to identify common regulatory mechanisms
55 across species. It does appear though, that besides the inhibition of the ribosome and the DNA
56 gyrase, little consistency exists in the transcriptional responses of different bacteria to the same
57 antibiotic class, indicating that to some extent the response to an antibiotic attack is species-specific.
58 Therefore, when querying a compendium the profiles of the compendium antibiotics and the novel
59 compound should have been generated using the same organism.

60 Bovine shipping fever is a severe inflammation of the bovine lung caused primarily by the gram
61 negative bacteria *Mannheimia haemolytica* and *Pasteurella multocida* (Mosier, 1997). Despite the
62 success of antibiotic treatment, the disease is far from controlled in feedlots (Duff & Galyean, 2007),
63 warranting the development of more efficient drugs. In order to aid the development of novel
64 compounds to treat shipping fever, we created a compendium of transcriptional profiles in *P. multocida*
65 to commonly used antibiotics in bovine pneumonia and queried it with the transcriptional profile of a
66 novel compound with unknown MoA and excellent activity on *P. multocida*.

67

68 **2. Materials and methods**

69 *Bacterial strains, antibiotics and growth conditions*

70 *Pasteurella multocida* L386 is a serovar A14 bovine isolate provided by Prof. Wieler of the Free
71 University of Berlin. *P. multocida* L386 was cultured on brain heart infusion (BHI, AES Laboratoire,
72 France) agar plates for 18 h at 37 °C. BHI broth cultures were incubated at 37°C with rotary aeration
73 at 220 rpm. Bacterial densities were determined by measuring the optical density (OD) at 578 nm. For
74 the microarray experiments growth was done as follows: for each antibiotic a mid-log grown culture
75 was split into 3 x 250 ml Erlenmeyer flasks to an OD₅₇₈ of 0.01 with a final volume of 40 ml BHI. One
76 flask served as non-treated control, the other two for the 10 and 30 min antibiotic-treated time points.
77 The antibiotics were added at minimal inhibitory concentration (1xMIC, Table 1) 10 and 30 min before
78 the untreated culture reached an OD₅₇₈ of ~ 0.5. Cells were harvested for five min at 5.000 g at 4°C
79 and were shock frozen and stored at -80 °C prior to RNA isolation. For each antibiotic and time point
80 at least three replicate cultures were prepared. Novobiocin sodium salt, enrofloxacin, florfenicol,
81 trimethoprim, rifampin and tilmicosin were purchased from Sigma-Aldrich (USA). The cefquinome
82 sulphate and the novel compound from the thiazin class were from Intervet Innovation, Germany.
83 Solutions for these antibiotics were prepared in H₂O. The trimethoprim, brodimoprim (Intervet
84 Innovation, Germany) and rifampin solutions were prepared in DMSO. The tilmicosin solutions were
85 prepared in phosphate buffer pH 7.2. Solutions were filter sterilized and used the same day. The MICs
86 of *P. multocida* L386 for the different antibiotics (Table 1) were determined using the standard micro-
87 broth dilution assay (National Committee for Clinical Laboratory Standards, 2003). See Table 1

88 *Microarray experiments & data analysis*

89 We used a custom-made Affymetrix antisense microarray containing 97% of the *P. multocida* Pm70
90 genome and 90 % of the *M. haemolytica* serovar A1 genome. The oligonucleotides on the microarray
91 were designed such that cross-hybridization was minimal. A total of 33 *M. haemolytica* gene-specific
92 oligonucleotides (1.2 % of the *M. haemolytica* genome) showed hybridization to cDNA from *P.*
93 *multocida*. The corresponding *P. multocida* orthologs were excluded from data analysis. Further
94 details concerning the microarray are described by Roehrig et al., 2007. RNA was isolated according
95 to standard protocols using QIAGEN RNeasy Midi columns (QIAGEN, Germany). The cDNA synthesis
96 started from 10 µg of RNA using the Superscript II reverse transcriptase (Invitrogen, USA) according
97 to the manufacturer's protocol. 3 µg of cDNA were fragmented into ~ 200 bp using 1.8 U of DNase I in
98 a 10 min reaction at 37 °C. Terminal biotin-dUTP labelling was done using the Enzo labelling kit
99 (Enzo, USA). All procedures for hybridization, washing and staining are described at

100 <http://www.affymetrix.com/support/technical/manual/expressionmanual.affx> for prokaryotic
101 microarrays. The hybridization signals were normalized using the Affymetrix Microarray Suite 5.0
102 software. A description of the experimental design and all data have been deposited in NCBI's Gene
103 Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series
104 accession number GSE10051. Replica quality was assessed by pair-wise comparisons of signal
105 intensities. We adhered to Affymetrix criteria which states that replicates are of good quality when less
106 than 3 % of all transcribed genes differ ≥ 2 -fold in signal intensity between two replicates. For each
107 treatment and time point at least three replicates fulfilling this requirement were generated to calculate
108 mean values. Statistical analysis and \log_2 transformation were done using the Vector Xpression
109 software (Invitrogen, USA). Only genes with a significant difference in transcription between control
110 and antibiotic-treated samples ($p=0.1$, Mann-Whitney test) and a ≥ 1.5 fold difference to control were
111 considered regulated.

112 *Real time PCR*

113 Real-time PCR using an ABI 7000 Sequence Detection System (Applied Biosystems, USA) was
114 performed to confirm the microarray-derived differences in gene transcription for some positive,
115 negative and non-regulated *P. multocida* genes. Primers (supplementary material, Table S1) were
116 designed using Primer Express 2.0 (Applied Biosystems, USA). The number of target molecules was
117 calculated using a standard curve obtained by real time PCR with titrations of gene-specific standard
118 templates ($10^2 - 10^8$ template molecules). We used the transcript levels of *recA* to account for
119 pipetting errors. *RecA* transcription did not alter between antibiotic-treated and control samples, with
120 the exception of enrofloxacin, and has previously been used to calculate transcriptional regulation
121 (Roehrig et al., 2007). *TopA* was used as a reference gene for the calculation of enrofloxacin-induced
122 transcription because *recA* was regulated by enrofloxacin. Quantification was done as follows: copy
123 number of the target gene in the antibiotic-treated sample / copy number of the target gene in the
124 control sample divided by the copy number of the reference gene *recA* in the antibiotic-treated sample
125 / the copy number of the reference gene *recA* in the control sample. Three individual reactions for
126 target genes and the reference genes were used to calculate mean values.

127

128 *Electronmicroscopy*

129 The effect of antibiotics on capsule expression was evaluated by electron microscopy. Bacterial
130 cultures were incubated for 3 h starting with an OD₅₇₈ of 0.15 in the presence of 0.5xMIC of
131 novobiocin, enrofloxacin, trimethoprim and florfenicol and without antibiotic for control purposes.
132 Bacteria were prepared for transmission electron microscopy following polycationic ferritin labelling as
133 described by McKerral & Lo (2002). Thin sections were stained with uranyl acetate and the capsular
134 thickness was determined using a Zeiss EM 109 electron microscope. The capsule thickness was
135 determined for ≥ 80 cells for control and antibiotic-treated cells. A Students two-sided t-test for two
136 independent samples was used to test the difference in capsule expression between untreated and
137 treated cells ($p < 0.05$).

138

139 **3. Results**

140 *Generation of a compendium of transcription profiles*

141 In order to create a database of *P. multocida* transcriptional profiles to antibiotic treatment, we
142 selected six commonly and two less commonly used antibiotics to treat bovine pasteurellosis (Table
143 1). The eight antibiotics inhibit processes representing the five most important MoAs: cell wall and
144 folate biosynthesis, DNA replication (DNA gyrase), translation (ribosome) and transcription (RNA
145 polymerase). The transcriptional profile of a new compound from Intervet's anti-infectives program with
146 excellent activity against *P. multocida* was also recorded. This compound is a heterocycle and a
147 derivative of the thiazin chemical class and will be referred to as thiazin. The cellular target of the
148 thiazin is unknown. We included the novel compound in this study to obtain first indications about its
149 putative MoA by comparing its transcriptional profile with the profiles from eight antibiotics with known
150 MoAs. So far, no bovine *P. multocida* isolate has been sequenced, precluding the construction of a
151 microarray covering the genome of a strain causing shipping fever. Therefore, we generated a
152 custom-made microarray representing 97% of all genes from an avian *P. multocida* serovar A strain
153 (May et al., 2001). The microarray was hybridized with the cDNA of a pathogenic bovine serovar A
154 strain. This meant that genes present in the bovine isolate, but absent in the avian isolate that was
155 used to construct the microarray, were missed. Despite the differences between the *P. multocida*
156 strains it is unlikely that strain-to-strain variations compromised the data. When the microarray was
157 hybridized with labelled genomic DNA from the bovine isolate, 94% of all printed genes from the avian
158 strain were detected; indicating that strain-to-strain variation probably is limited to about 6%. For the
159 antibiotic assault, the bovine *P. multocida* serovar A strain was treated for 10 and 30 min with 1xMIC

160 (Table 1) of each antibiotic. The eight antibiotics and the novel compounds caused > 1,5 fold
161 regulation of 1468 genes (Table S2 in the supplementary material for $\log_2(\text{antibiotic-treated/control})$
162 ratios for all genes showing regulation ≥ 1.5 fold). For a subset of genes the microarray data were
163 verified by real time PCR. Fig.1 shows that the microarray-derived relative transcription levels
164 correlated well with the relative transcription levels obtained by real time PCR.

165 *Global transcription analysis*

166 A first impression of the similarities and differences of the transcriptional profiles was obtained by
167 clustering the expression profiles into a hierarchical tree (Fig. 2). The transcriptional profiles of the two
168 time points for each antibiotic were most similar and hence located closely together on the hierarchical
169 tree. The two dihydrofolate reductase (DHFR) inhibitors trimethoprim and brodimoprim and the two
170 ribosomal inhibitors tilmicosin and florfenicol each formed single expression clusters, supporting the
171 notion that antibiotics with related MoAs produce similar expression profiles (Hutter et al., 2004). An
172 exception to this rule was observed for the two DNA gyrase inhibitors enrofloxacin and novobiocin,
173 which produced very different expression profiles and thus located apart on the hierarchical tree (Fig.
174 2). The enrofloxacin-induced transcription profile was most similar to the transcription profiles
175 produced for cefquinome and the thiazin despite the differences in chemical structures. The antibiotics
176 differed substantially in their impact on the number of regulated genes. The difference was most
177 evident at the early (10 min) time point when secondary transcriptional responses were less apparent
178 (Table 2). Novobiocin caused a massive alteration in gene transcription with nearly 40% of all *P.*
179 *multocida* genes affected. Florfenicol, tilmicosin, rifampin, trimethoprim and brodimoprim changed the
180 transcription of 10 to 20% and enrofloxacin, cefquinome and the thiazin altered the expression in the
181 order of 1 % of all genes. To unveil further global differences between the antibiotics, the early
182 regulated genes were assigned to 14 functional groups (Fig. 3). We limited the analysis to the early
183 time point in order to reduce the influence of secondary responses. The transcription of genes
184 involved in protein fate, transport and binding as well as energy metabolism were affected most
185 markedly by all antibiotics. The functional classification revealed similarities but also striking
186 differences between the antibiotics, even between antibiotics inhibiting the same target: for instance
187 novobiocin and enrofloxacin which both inhibit the DNA gyrase. While novobiocin stimulated the
188 regulation of > 25% of the genes in all functional groups, with an even stronger impact (> 50%) in the
189 functional groups transcription, protein fate and cofactor biosynthesis, enrofloxacin only regulated
190 genes in less than half of the 14 categories and only to 1-4 % (Fig. 3). Likewise, trimethoprim caused

191 two to four fold more regulation than brodimoprim in the functional groups amino acid, cofactor and
192 protein synthesis (Fig. 3). The two ribosomal inhibitors florfenicol and tilmicosin had a similar impact
193 on gene regulation in all but fatty acid and phospholipid metabolism, where florfenicol regulated 6 %
194 whilst tilmicosin regulated 26% of all genes. Rifampin caused regulation in all functional groups to an
195 extent that was surprisingly similar to florfenicol up to the point that both antibiotics regulated the same
196 percentage of genes in the categories cell wall synthesis and fatty acid and phospholipid metabolism
197 (Fig. 3), albeit not the same genes (Table S1 supplementary material).

198 *Regulation of target and signature genes*

199 The global analysis unveiled substantial differences in gene regulation between antibiotics with similar
200 structure or related MoA. This observation was supported when the regulation of target genes was
201 analysed. Novobiocin induced two to three fold the transcription of genes coding for the DNA gyrase
202 subunits, *gyrAB* and down-regulated the transcription of the topoisomerase I (*topA*) and *parEC* genes
203 coding for the subunits of the topoisomerase IV (Table 3 and Table S2 supplementary material).

204 Transcription of the gene PM0842 downstream of *gyrA* (PM0841) was also induced by novobiocin
205 (Table S2 supplementary material). PM0842 codes for a regulator with similarity (41% similarity and
206 27 % identity on protein level) to *sirB* involved in the regulation of *Salmonella enterica* serovar
207 Typhimurium virulence genes. Novobiocin inhibits the ATPase function of the GyrB subunit of the DNA
208 gyrase by competing with ATP for the ATP binding pocket (Lewis et al., 1996). The down-regulation of
209 35 and the up-regulation of 22 ATPases-encoding genes is in line with the inhibition of ATP binding by
210 novobiocin (Table S2 supplementary material). Many of the regulated genes coded for ABC-type
211 transporters involved in the uptake of nutrients, vitamins and ions. A comparable broad regulation of
212 ATPases was not observed for the other antibiotics and therefore was considered a signature
213 response for novobiocin (Table 3). A significant portion of novobiocin-regulated genes were not
214 regulated by any of the other antibiotics. For instance novobiocin repressed a region on the *P.*
215 *multocida* chromosome comprising of seven genes (PM0469-PM0476). Not all of these genes were
216 exclusively regulated by novobiocin, but the early onset after 10 min and the negative direction of
217 regulation was specific for novobiocin (Table 3, Table S2 supplementary material). The region
218 comprises of a putative ABC-type periplasmic transport complex encoded by three genes (PM0470,
219 PM0471, PM0472) and a large ORF (876 amino acids) coding for a potential *TRAP*-type transporter
220 (PM0473). Enrofloxacin had no influence on *gyrAB* and *parEC* target gene transcription (Table 3,
221 Table S2 supplementary material) or on *topA* transcription. Enrofloxacin blocks the *GyrA* subunit

222 leading to the stabilization of single stranded DNA ends during DNA unwinding (Walsh, 2002).
223 Indicative of this effect was the induction of nine genes coding for proteins involved in the repair of
224 DNA, like the *lexA* and *impA* repressors, the *recX* regulator and *recA* (Table S2 supplementary
225 material). *RecA* and *impA* transcription was not changed in the presence of the other antibiotics, only
226 florfenicol increased *recA* transcription to slightly above the cut-off value at the 30 min time point.
227 Therefore, *recA* and *impA* up-regulation was considered indicative of enrofloxacin treatment (Table 3).
228 The ribosomal inhibitors florfenicol and tilmicosin had a very similar impact on transcription.
229 Approximately 55% of all florfenicol- and tilmicosin-regulated genes were regulated by both antibiotics.
230 With respect to the ribosomal target, florfenicol up-regulated 14 ribosomal genes and tilmicosin up-
231 regulated 14 and down-regulated one ribosomal genes. Of all other antibiotics, only rifampin had a
232 comparable influence on ribosomal gene transcription, albeit of the 15 rifampin-regulated ribosomal
233 genes the majority (9) were down-regulated. Despite the similarities, some genes were exclusively
234 regulated by either florfenicol or tilmicosin and were not regulated by any of the other antibiotics. For
235 instance florfenicol caused a strong (9 -16 fold) up-regulation of a hypothetical gene of unknown
236 function (PM1129) and tilmicosin caused a strong up-regulation of PM0836, another hypothetical gene
237 of unknown function (Table 3).
238 The two DHFR inhibitors trimethoprim and brodimoprim are identical except that the 4-methoxy group
239 of the benzyl ring of trimethoprim is substituted by bromine in brodimoprim. Despite the structural
240 relatedness only 40 % of all 506 trimethoprim- and brodimoprim-regulated genes were regulated by
241 both antibiotics. The transcription of the dihydrofolate reductase-encoding (*folA*) target gene was not
242 influenced by both DHFR inhibitors (Table 3), nor did any of the other antibiotics influence *folA*
243 transcription. Trimethoprim weakly induced *foIP* coding for the dihydropteroate synthase target of the
244 sulfonamides (Table S2 supplementary material), but otherwise trimethoprim and brodimoprim exerted
245 no transcriptional impact on the folic acid biosynthesis pathway. Regulation of signature genes was
246 also observed for both DHFR inhibitors (Table 3). Trimethoprim up-regulated the transcription of
247 PM1190, coding for a putative zinc-dependent protease, brodimoprim specifically up-regulated a
248 hypothetical gene of unknown function (PM1127).
249 The RNA polymerase inhibitor rifampin inhibits the activity of the RNA polymerase by binding to the
250 β subunit of the pentameric $\alpha_2\beta\beta'\omega$ RNA polymerase core complex (Walsh, 2002). Rifampin
251 moderately up-regulated the expression of the β and β' subunits encoded by the *rpoB* and *rpoC* genes,
252 respectively (Table 3), but had no influence on the expression of the α and ω subunits encoded by the

253 *rpoA* and *rpoZ* genes, respectively. Consistent with its influence on transcription, rifampin altered the
254 transcription of genes influencing sigma factor expression. The σ^{70} sigma factor encoded by *rpoD*
255 gene was induced approximately two fold concomitant to an increase in transcription of two genes
256 involved in the regulation of σ^{70} , the anti-sigma factor B antagonist (PM0178) and the putative sigma
257 factor regulatory gene (PM0092, Table S2 supplementary material). Transcription of *rpoE* coding for
258 the σ^E factor was down-regulated approximately two fold at 30 min, which coincided with the
259 simultaneous down-regulation of the σ^E regulatory protein encoded by *resB* (Table S2 supplementary
260 material). Most of the rifampin-specific genes were up-regulated and coded for hypothetical proteins of
261 unknown function, e.g. the putative operon PM1679-1682 (Table 3).

262 Of all antibiotics, cefquinome had the smallest influence on gene transcription with only seven
263 regulated genes at the 10 min time point and another 70 genes at the 30 min time point (Table S2
264 supplementary material). None of the six putative penicillin-binding target protein-encoding genes
265 were regulated and no cefquinome-specific signature gene meeting the minimum signature gene
266 requirements of \geq two fold regulation at both time points was identified (Table 3). The low impact on
267 global gene regulation was accompanied by a small impact on the magnitude of regulation. The most
268 strongly cefquinome-regulated gene was *mgIB* coding for a periplasmic sugar transporter with a two
269 fold increase and PM1895, coding for a hypothetical protein, with an approximately three fold
270 decrease in expression (Table S2 supplementary material). The weak impact of cefquinome on *P.*
271 *multocida* gene regulation was further illustrated by the short duration of regulation. Only one gene,
272 *hbpA* coding for the heme binding protein A, was negatively regulated at both time points, all other
273 regulated genes were only affected either at 10 min or at 30 min.

274 Similar to the other bactericidal compounds enrofloxacin and cefquinome, the thiazin with an unknown
275 MoA exerted only a minor impact on gene regulation. Just 27 genes were regulated at 10 min of which
276 all were repressed except *artM* which is involved in arginine transport (Table S2 supplementary
277 material). Unlike for all other antibiotics, the thiazin did not induce a broad secondary response, just
278 four genes were regulated at 30 min. The four late genes differed from the early regulated genes,
279 indicating that the thiazin effect on transcription diminished quickly. With the exception of *artM*, all
280 other thiazin-regulated genes were also regulated by one or several of the other antibiotics and
281 belonged predominantly to the highly regulated functional groups energy metabolism and transport
282 and binding. Conclusively, the transcriptional response to thiazin treatment did not provide indications
283 of the inhibited target(s).

284 *Regulation of drug transporters*

285 Many bacteria respond to antibiotic treatment with the induction of resistance-conferring genes. *P.*
286 *multocida* Pm70 contains a number of resistance-conferring genes on the chromosome which are
287 present on the microarray (plasmid-located resistance genes are absent on the microarray). The
288 ability of *P. multocida* to respond to antibiotic treatment through the regulation of drug transporters
289 was seen in the cases of novobiocin, florfenicol and tilmicosin where the *acrAB* genes involved in the
290 extrusion of antibiotics were induced (Ma et al., 1993) (Table S2 supplementary material). A putative
291 *tetR*-like regulator (PM1135) of *acrAB* transcription was also induced by novobiocin. The *AcrAB*
292 proteins form a pore together with *ToIC*, which was not regulated. A hypothetical gene (PM1581) with
293 weak homology to *marC* of *E. coli* (25% protein identity) was induced by the two ribosomal and the
294 two DHFR inhibitors (Table S2 supplementary material). *MarC* codes for an integral membrane protein
295 of the multidrug-resistance (MDR) family of transporters (Aleksun & Levy, 1999). Other genes
296 belonging to a cluster of orthologous groups (COG0697) involved in drug transport were up-regulated
297 by florfenicol, tilmicosin, trimethoprim and brodimoprim (Table S2 supplementary material).
298 Interestingly, the bactericidal antibiotics enrofloxacin, cefquinome and the thiazin did not alter any of
299 the *P. multocida* chromosomally located resistance-associated genes presented on the microarray.

300 *Regulation of virulence genes*

301 The transcription of many of the known and putative *P. multocida* virulence factors was influenced in
302 the presence of the antibiotics. The bacteriostatic antibiotics with a strong impact on global gene
303 regulation (Table 2) affected virulence gene transcription much more than the bactericidal antibiotics
304 cefquinome, enrofloxacin and the thiazin, which hardly influenced virulence gene regulation. Table 4
305 summarizes the impact on virulence gene transcription by the six bacteriostatic antibiotics. The
306 virulence factors were selected from the *P. multocida* genome annotation by May et al. (2001) and
307 Ewers et al. (2004). Virulence gene transcription was predominately negatively affected, e.g. tilmicosin
308 down-regulated 32 and up-regulated seven virulence genes. Only rifampin up-regulated more
309 virulence genes (24) than down-regulated (10). The synthesis and transport of capsular
310 polysaccharides and LPS, the transport of iron, adherence and competence were the most strongly
311 affected virulence factors. With respect to iron transport, the genes coding for the critical *tonB* *exbBD*
312 system providing the energy for many iron transport systems (Braun, 1995) was intermediately (2-7
313 fold) repressed by all bacterostatic antibiotics (Table 4). Furthermore, the ferric-binding protein *fbpA*,
314 the *hugZ* gene involved in heme detoxification and the *afuABC* genes involved in ferric transport were

315 repressed by novobiocin, florfenicol and tilmicosin. Transcription of the iron regulator *fur* was weakly
316 induced by novobiocin. *P. multocida* expresses two forms of a filamentous hemagglutinin, *PfhB1* and
317 *PfhB2* involved in adherence. The transcription of both *pfhB* genes was moderately down-regulated by
318 novobiocin, florfenicol and tilmicosin. Secretion of the haemagglutinins is mediated by the *IspB1* and
319 *IpsB2* gene products. *LspB1* transcription was down-regulated by novobiocin and tilmicosin. The
320 *tadABCDEF* locus involved in tight adherence (Tomich et al., 2007) was up-regulated by rifampicin.
321 The broad induction of the *tad* locus was specific for rifampin, all other antibiotics had no or only a
322 minor impact on *tad* regulation. Transcription of the competence locus involved in DNA uptake was
323 negatively affected by several antibiotics. The *P. multocida* competence genes are organized in a
324 *comABCD* operon and two genes (*comM*, *comF*) located elsewhere on the chromosome.
325 Trimethoprim down-regulated the expression of the *comABCD* operon at both time points, tilmicosin
326 only at 30 min. Novobiocin strongly repressed *comM* and to a lesser extent *comF* transcription. The
327 *P. multocida* LPS is a major virulence factor in sepsis (Harper et al, 2007a; 2007b). The transcription
328 of 16 genes involved in the synthesis and modification of LPS was positively and negatively altered.
329 For instance, novobiocin down-regulated the *galE* gene coding for the galactose epimerase involved in
330 the synthesis of the LPS core and O-chain. Conversely, *glpQ* was up-regulated by novobiocin. GlpQ
331 is responsible for the transfer of choline to the LPS, which is important for *P. multocida* virulence
332 (Harper et al., 2007a). *P. multocida* contains 18 genes involved in the metabolism and transfer of sialic
333 acids. Sialic acid uptake and metabolism could play a role in *P. multocida* virulence by providing
334 carbohydrate nutrient and for decorating LPS with the help of sialyltransferases for immune evasion
335 (Steenbergen et al., 2005). The two divergently transcribed sialic acid operons harbouring the genes
336 necessary for transport (*nanP*, *nanU*) and metabolism (*nanA*, *nanK*, *nanE*) of host-derived sialic acid
337 were down-regulated by novobiocin. Amongst the down-regulated genes was also PM1714 coding for
338 a putative regulator of the two operons. Florfenicol and tilmicosin repressed the transcription, whilst
339 rifampin induced the transcription of some genes of the sialic acid locus. Other up-regulated virulence
340 genes were involved in the response to stress conditions, e.g. the catalase-encoding gene *hktE* and
341 the *sodC* gene coding for the superoxid dismutase, both were positively regulated by florfenicol,
342 tilmicosin and novobiocin.

343 The capsule of *P. multocida* is an important virulence factor for successful host colonization (Chung et
344 al., 2001). The transcription of nine capsule biosynthesis and transport genes was strongly down-
345 regulated by novobiocin, florfenicol, tilmicosin, trimethoprim and brodimoprim (Table 4). Of all these

346 antibiotics novobiocin affected capsule gene transcription most strongly with a 4-16 fold repression of
347 all capsule genes at both time points, whilst the DHFR inhibitors affected capsule gene transcription
348 predominately at the 30 min time point (Table 4). We were intrigued to verify whether the repression of
349 the capsule genes correlated with a decrease in capsule expression on the surface of *P. multocida*. *P.*
350 *multocida* was grown in absence and presence of antibiotics and capsule expression was quantified
351 by measuring the thickness of the capsular layer by electron microscopy. We assumed that a
352 phenotypic effect would be best visible after several cell divisions; therefore the antibiotic
353 concentration was reduced to 0.5xMIC to permit growth. To account for growth effects on capsule
354 expression, *P. multocida* was treated with enrofloxacin, which did not alter the transcription of capsule
355 genes (Table S2 supplementary material) and hence was expected not to affect the level of
356 capsulation. Fig. 4 shows the quantification of capsulation from electron microscopic pictures
357 exemplified in Fig. 5. In the absence of antibiotics approximately 65% of *P. multocida* cells displayed a
358 capsular layer greater 30 nm thickness, about 30 % showed an intermediate level of capsulation (< 30
359 nm) and the rest expressed no or hardly capsulation. Novobiocin caused a significant ($p < 0.05$)
360 disappearance of thick capsules > 30 nm and a concomitant increase in the fraction of cells with an
361 intermediate level of capsulation (Fig. 4). Trimethoprim also caused a significant decrease in the
362 fraction of cells with high and intermediate levels of capsulation and a concomitant increase of cells
363 with no capsules (Fig. 4). Florfenicol did not significantly ($p < 0.05$) alter capsule expression as
364 compared to untreated cells, albeit a trend to reduced capsulation was visible. The florfenicol-induced
365 distribution of capsulation resembled that after enrofloxacin treatment, which also did not affect the
366 level of capsulation as compared to untreated cells. Conclusively, a general correlation between
367 antibiotics reducing capsule gene transcription and a phenotypic response on the level of capsulation
368 was not observed.

369

370 4. Discussion

371 Only few of the eight compendium antibiotics with known MoA induced transcription profiles in *P.*
372 *multocida* indicative of the cellular target. This was the case for the ribosomal inhibitors tilmicosin and
373 florfenicol which caused a strong up-regulation of ribosomal genes, clearly reflecting a compensatory
374 mechanism to ribosomal inhibition. Ribosomal gene regulation is consistent with earlier reports for the
375 ribosomal inhibitors chloramphenicol and erythromycin in *Bacillus subtilis* and *Streptococcus*
376 *pneumoniae*, respectively (Lin et al., 2005; Ng et al., 2003). The ribosomal target regulation was made

377 apparent by the high number of regulated ribosomal genes, which allowed easy detection amongst the
378 other regulated non-target proteins. Regulation of targets encoded by one or only few genes was less
379 obvious, as was the case for rifampin and novobiocin. Rifampin caused regulation of the *rpoBC* RNA
380 polymerase subunit-encoding genes and left a specific impact on accessory genes, like the σ^{70} and
381 σ^E -encoding *rpoD* and *rpoE* genes and their putative regulators *resB*, PM0092 and PM0178. Induction
382 of *rpoB* by rifampin has also been observed in *E. coli* (Shaw et al., 2003). However, the regulation of
383 transcription factors was not specific to rifampin, e.g. novobiocin also caused the up-regulation of the
384 *rpoBC* RNA polymerase genes and sigma factors. Novobiocin provided indirect evidence to its GyrB
385 target by the regulation of many genes coding for ATPases, mostly ABC-type transporters, some with
386 critical functions in the uptake of vitamins and nutrients. The regulation of the many ATPases by
387 novobiocin is probably associated with its ability to occlude the conserved ATP binding site of the
388 GyrB subunit (Lewis et al., 1996) and therefore may not be an exclusive reaction to novobiocin. The
389 regulation of *gyrAB* transcription by novobiocin and the lack of regulation by enrofloxacin were similar
390 to what has been observed in *H. influenzae*, with the exception that the *gyrA* subunit was only induced
391 at high concentrations in *H. influenzae* (Gmuender et al., 2001). The up-regulation of *gyrAB* by
392 novobiocin was concomitant to a strong down-regulation of the topoisomerase I (*topA*) and an
393 intermediate down-regulation of topoisomerase IV (*parEC*). TopA relaxes negatively supercoiled DNA,
394 whilst GyrAB induces negative turns. Thus, the down-regulation of *topA* transcription could be a
395 compensatory response to the inhibition of GyrAB. Enrofloxacin left a transcriptional trace to its MoA
396 by the induction of SOS response genes. The SOS response was most likely triggered by the
397 stabilization of single stranded DNA during *GyrAB* inhibition, an effect also observed for
398 fluoroquinolones in other bacteria (Gmuender et al., 2001, Shaw et al., 2003, Kaldalu et al., 2004). No
399 direct or indirect indications on the MoA were obtained from the transcriptional profiles of the DHFR
400 inhibitors trimethoprim and brodimoprim and the inhibitor of cell wall transpeptidases cefquinome. The
401 lack of target gene regulation by cefquinome was not due to a general insensitivity of the penicillin-
402 binding target proteins to regulation, as novobiocin, florfenicol and trimethoprim altered the
403 transcription of several of these proteins. A lack of transpeptidase regulation by β -lactams has also
404 been observed in *E. coli* (Shaw et al., 2003, Kaldalu et al., 2004).

405 It was obvious that the bactericidal drugs enrofloxacin, cefquinome and the thiazin regulated few
406 genes, whilst the bacteriostatic drugs florfenicol, tilmicosin, trimethoprim and brodimoprim had an
407 intermediate and novobiocin a strong impact on global gene regulation. Previous studies with

408 bactericidal and bacteriostatic compounds (Shaw et al., 2003, Kaldalu et al., 2004, Freiberg et al.,
409 2005) are difficult to compare with our study because of the differences in antibiotic concentrations,
410 time points and cut-offs for defining regulation. The expression profiling study by Gmuender et al.
411 (2001), which is most closely related to our study, did not report an explicit difference in the number of
412 regulated *H. influenzae* genes after novobiocin (bacteriostatic) and ciprofloxacin (bactericidal)
413 treatment, therefore this phenomenon may be specific to *P. multocida*. RNA yield and the transcription
414 levels of control genes were unchanged in the presence or absence of bactericidal compounds, ruling
415 out technical reasons. A principal difference between bacteriostatic and bactericidal compounds is the
416 recently discovered death pathway induced by bactericidal but not by bacteriostatic compounds
417 (Kohanski et al, 2007). The death pathway is triggered by the depletion of NADH and the subsequent
418 liberation of ferrous iron which in turn gives rise in toxic hydroxyl radicals via the Fenton reaction.
419 Depletion of NADH levels by bactericidal compounds was related to the increase in NADH
420 dehydrogenase I expression compared to cells treated with the bacteriostatic drug spectinomycin
421 (Kohanski et al, 2007). In *P. multocida* this enzyme complex (PM1329-1333) was not regulated by the
422 bactericidal drugs enrofloxacin, cefquinome and the thiazin. As an alternative to an explanation
423 involving the death pathway, the difference in gene regulation between bactericidal and bacteriostatic
424 compounds may be target-dependent. It could be that the inhibition of the DNA gyrase and the
425 transpeptidases by the bactericidal compounds enrofloxacin and cefquinome, respectively, is of lower
426 transcriptional consequence because inhibition of these targets doesn't affect the energy metabolism
427 of the cell as much as the inhibition of the bacteriostatic targets, e.g. the inhibition of folate
428 biosynthesis by trimethoprim.

429 The observed antibiotic effects on virulence gene transcription and expression adds to a growing body
430 of evidence indicating that antibiotics block virulence gene expression. Examples are the effect of
431 azithromycin on *Pseudomonas aeruginosa* quorum sensing and biofilm formation (Nalca et al., 2006)
432 and the down-regulation of invasion and flagellar genes in *Salmonella typhimurium* by polymyxin
433 (Bader et al., 2003). However, the inverse effect on virulence, the stimulation of biofilm formation and
434 type III secretion expression by antibiotics has also been observed (Linares et al., 2006). In our study
435 the antibiotics repressed virulence gene transcription with the exception of rifampin. Many of the
436 regulated virulence genes, like the competence genes, the capsule genes and the *tad* genes involved
437 in tight adherence, are located in operons, explaining their coordinated regulation. The transcription of
438 some major virulence factors was negatively affected; for example: iron transport, adhesion,

439 competence, LPS biosynthesis and capsule biosynthesis and export. However, a cautionary note must
440 be raised concerning a generalized conclusion based only on in vitro transcriptional data. First,
441 antibiotic gene regulation was studied in the nutrient-rich environment of bacteriological growth media
442 and not in the complex and challenging environment of the infected host, where many of the virulence
443 genes are regulated differently. Second, the experimental conditions entailed that an antibiotic-
444 induced repression of those virulence genes which are not transcribed under standard growth
445 conditions was missed. For instance, many iron transporters are repressed under the iron-replete
446 growth conditions used in this study, but are induced when the bacterium faces iron limitation, as it is
447 the case during an infection. In our study only antibiotic-induced induction, but not repression, of these
448 virulence genes would have been observed. Still, the *tonB exbDB* system was transcribed enough to
449 detect antibiotic-induced repression even under the iron-replete conditions. Third, the phenotypic
450 impact of antibiotic transcriptional regulation remains uncertain for most genes. A two fold difference in
451 mRNA levels could result in a strong phenotypic effect for some but no phenotypic effect for other
452 virulence genes. We analysed the phenotypic effect on capsule repression and found a relationship
453 between transcriptional down-regulation and a thinning of the capsule on the surface of *P. multocida*
454 for novobiocin and trimethoprim. However, a general correlation between repression of capsule gene
455 transcription and capsule thickness was not confirmed. Florfenicol did not cause a thinning of the
456 capsular layer although the repression of capsule gene transcription exceeded that of trimethoprim.
457 This result does not exclude a contribution of capsule gene repression to the observed reduction in
458 capsule surface expression, but demands prudence when interpreting transcriptional alterations into
459 phenotypic effects. The depletion of glucuronic acid and N-acetylglucosamine precursor molecules for
460 the hyaluronic acid capsule, caused by secondary effects of novobiocin and trimethoprim on the
461 metabolism of sugar molecules, may also explain the reduction in capsule surface expression. It is
462 interesting that so many bacteriostatic antibiotics affected capsule gene transcription, pointing to a
463 common, MoA-independent regulatory mechanism, affecting either the promotor activity of the capsule
464 operon or capsule mRNA stability.

465 The compendium of antibiotic-induced transcriptional profiles has become a useful basis for MoA
466 predictions in our drug discovery program. Albeit, the very different transcriptional profiles of
467 novobiocin and enrofloxacin which inhibit the same DNA gyrase target illustrate that transcriptional
468 profiling can only be one of many pieces needed to complete the MoA puzzle. At best it can provide a
469 first hypothesis, which must then be verified by biochemical and genetic approaches. Therefore, the

470 unique transcriptional profile of the thiazin does not confirm but provides first evidence for a new MoA.
471 However, with the rise of cross-resistance to existing antibiotics this information could become an
472 important trigger for drug development.

473

474 **Appendix A.** Supplementary data.

475

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549

550 **Legends to Figures**

551 Fig. 1. Verification of microarray data by real-time PCR.

552 \log_2 ratios (treated/control) were calculated from signals derived from microarray data (ordinate) and
553 real time PCR (abscissa). A total of five genes up- and down-regulated by four antibiotics were
554 analysed. r^2 denotes the correlation coefficient.

555

556 Fig. 2. Cluster analysis of transcriptional profiles.

557 Hierarchical clustering of relative expression values was performed using the complete linkage cluster
558 algorithm and euclidean distance similarity metric which places an emphasis on the magnitude of
559 expression values.

560

561 Fig. 3. Functional categorization of regulated genes.

562 The regulated genes 10 min after addition of the antibiotics were assigned to 14 functional groups
563 according to the annotation by May et al. (2001). The functional groups differed strongly in the number
564 of genes. Thus, the antibiotic impact was illustrated by presenting the percentage of regulated genes
565 per group.

566

567 Fig. 4. The effect of antibiotics on capsule expression.

568 *P. multocida* was grown to mid-log phase in the presence and absence of antibiotics at 0.5xMIC
569 concentrations (Table 1). Cells were stained with cationized ferritin, fixed and processed for electron
570 microscopy. The abscissa indicates the percentage of cells with > 30 nm (left bars), < 30 nm (middle
571 bars) capsule thickness and no capsule (right bars). Experiments were done in triplicate. A * indicates
572 a significant ($p < 0.05$, Students t-test) difference on the level of capsulation between untreated control
573 cells and antibiotic-treated cells.

574

575 Fig. 5. Surface expression of capsule polysaccharides in the presence of antibiotics.

576 *P. multocida* was grown to mid-log phase in the presence and absence of antibiotics at 0.5xMIC
577 concentrations. Cells were stained with cationized ferritin, fixed and processed for electron
578 microscopy. Non-treated control (A), novobiocin (B), florfenicol (C), trimethoprim (D). The bars indicate
579 200 nm.

Fig. 1

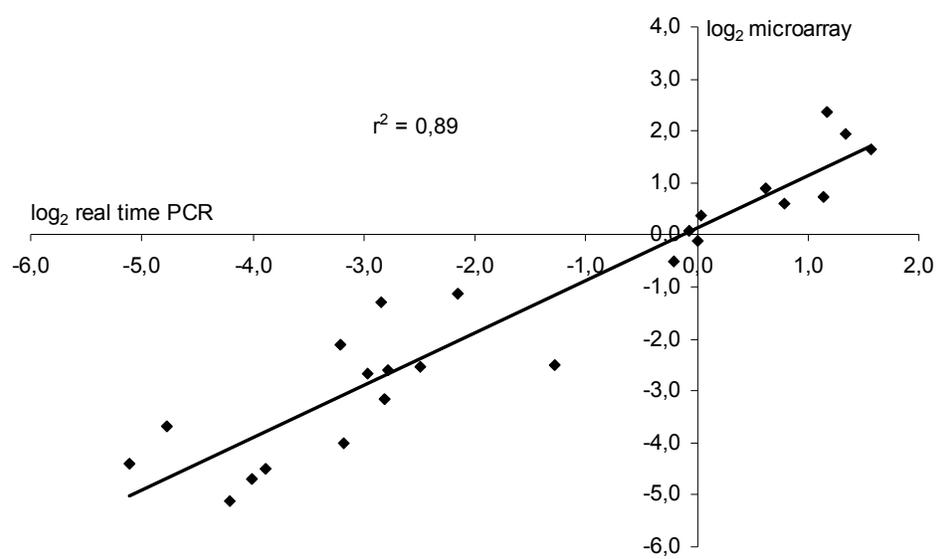


Fig. 2

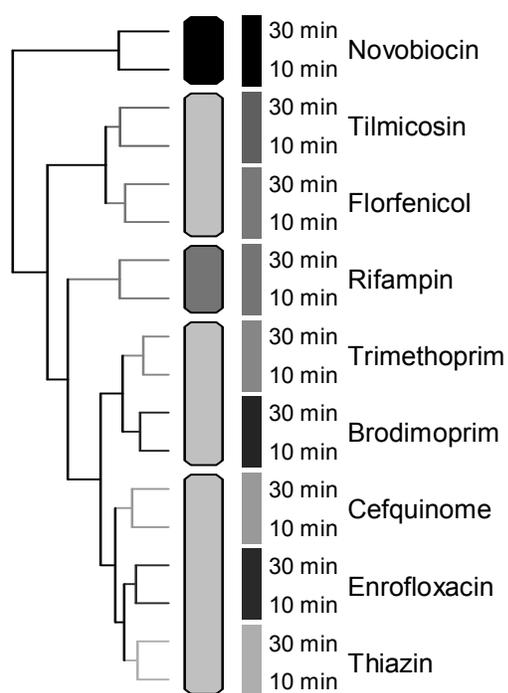


Fig. 3

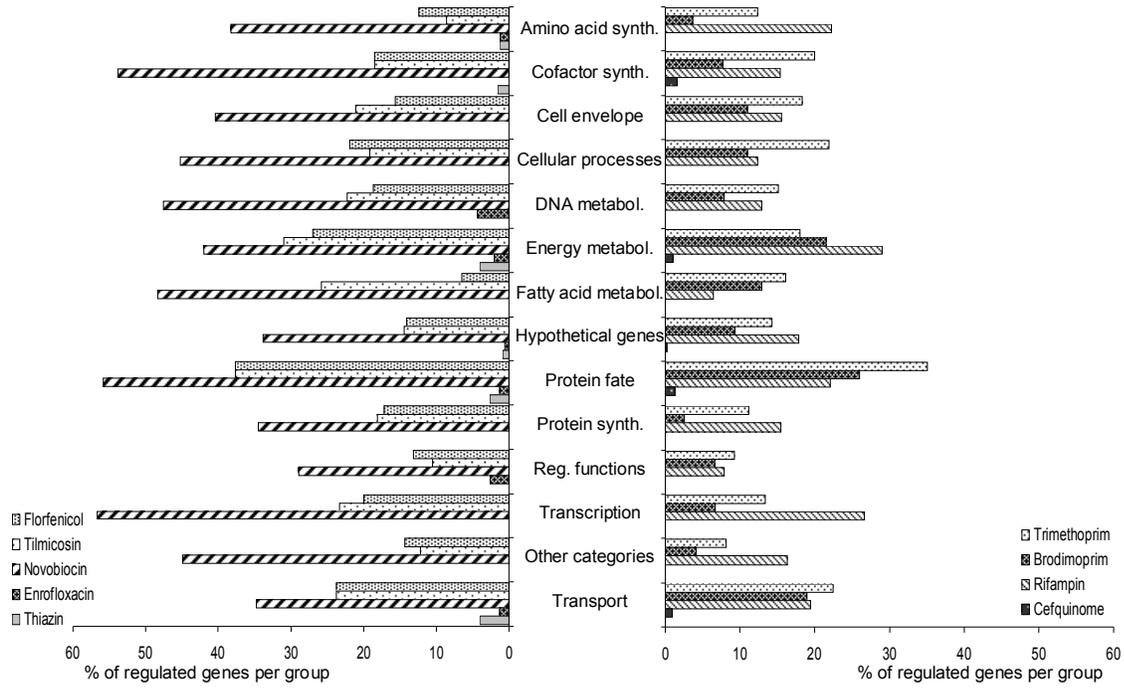


Fig. 4

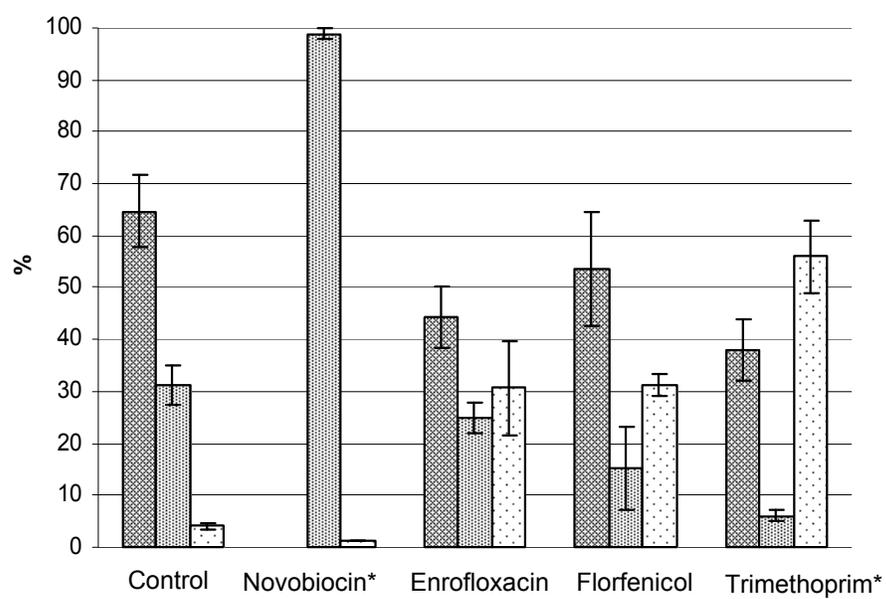


Fig. 5

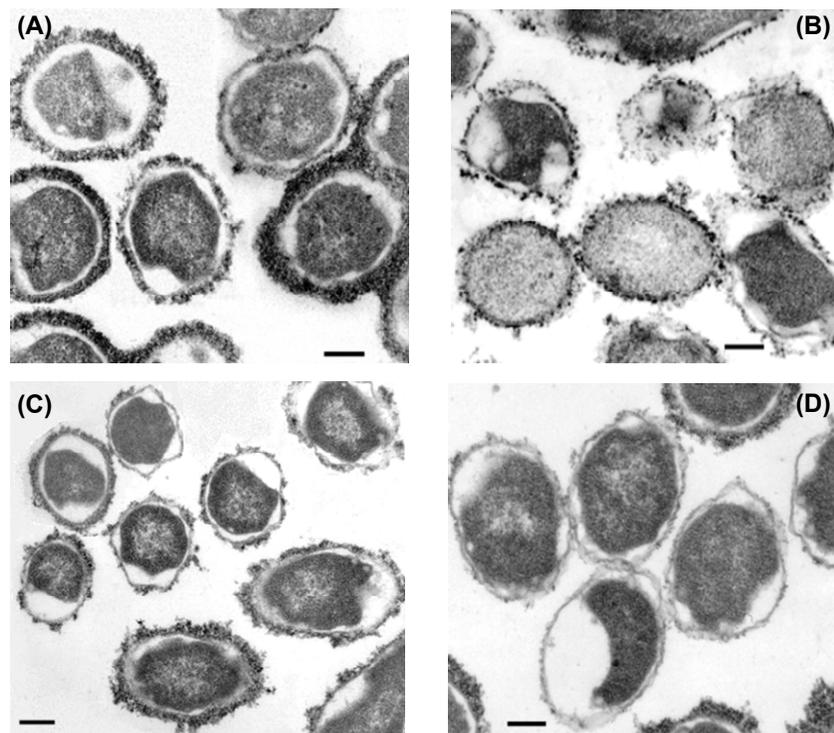


Table 1. MIC values for *P. multocida* serotype A bovine isolate

Antibiotic (substance class)	MIC ($\mu\text{g/ml}$)	MoA description
Enrofloxacin (quinolone)	0.016	inhibits the <i>gyrA</i> subunit of the DNA gyrase
Novobiocin (coumarin)	4.0	inhibits the ATPase function of the <i>gyrB</i> subunit of the DNA gyrase
Florfenicol (chloramphenicol)	0.5	blocks ribosome function by binding to the 50s subunit
Tilmicosin (macrolide)	4.0	blocks ribosome function by binding to the 50s subunit
Trimethoprim (diaminopyrimidine)	0.5	inhibits the dihydrofolate reductase (folate biosynthesis)
Brodinoprim (diaminopyrimidine)	0.25	inhibits the dihydrofolate reductase (folate biosynthesis)
Rifampin (rifamycin)	0.5	stalls transcription by inhibiting the β -subunit of the RNA polymerase
Cefquinome (β -lactam)	0.063	inhibits transpeptidases involved in peptidoglycan synthesis
Thiazin	0.031	unknown

Table 2: The number of regulated genes at 10 min

	up	down	% genome
Novobiocin	293	490	39,2
Florfenicol	189	185	18,7
Tilmicosin	168	235	20,2
Trimethoprim	160	176	16,8
Brodinoprim	113	119	11,6
Rifampin	234	131	18,3
Cefquinome	1	6	0,4
Enrofloxacin	10	11	1,1
Thiazin	1	26	1,3

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Table 3: Regulation of target and signature genes

Antibiotic	Target genes	Signature* genes
Novobiocin	<i>gyrAB</i> ↑	ABC-type transporters ↓↑ PM0469-0476 ↓
Enrofloxacin	<i>gyrAB, parEC</i> →	<i>recA</i> ↑ <i>impA</i> ↑
Florfenicol	ribosomal genes ↑	PM1129 ↑
Tilmicosin	ribosomal genes ↑	PM0836 ↑
Trimethoprim	<i>folA</i> →	PM1190 ↑
Brodimoprim	<i>folA</i> →	PM1127 ↑
Rifampin	<i>rpoBC</i> ↑	PM1679-1682 ↑, <i>wza</i> ↑
Cefquinome	<i>ponAB, dacAB, pbp2, ftsI</i> →	none
Thiazin	unknown	none

* signature genes were regulated > 2 fold at both time points. Expression values are presented in Table S2. Arrows indicate up-, down- and no regulation.

Table 4. The impact of antibiotics on *P. multocida* virulence gene regulation

Gene Name*	Description	Novobiocin		Florfenicol		Tilmicosin		Rifampin		Trimethop.		Brodimop.		Locus
		10'	30'	10'	30'	10'	30'	10'	30'	10'	30'	10'	30'	
phyB	Capsule biosyn.	-2,9	-2,1								-1,1		-0,7	PM0772
phyA	Capsule biosyn.	-3,5	-3,1			-1,5	-0,6	-1,3			-0,6	-1,2	-1,2	PM0773
hyaE	Capsule biosyn.	-4,3	-3,7	-0,8	-1,9	-1,2	-2,4					-1,2	-1,3	PM0774
hyaC	Capsule biosyn.	-3,3	-3,2	-1,0	-2,7	-1,6	-3,7					-1,2	-1,5	PM0776
hyaB	Capsule biosyn.	-3,0	-2,6	-0,9	-2,2	-2,5	-4,6	0,7	0,9	-0,7	-1,0	-1,0	-1,8	PM0777
hexD	Capsule transport	-2,8	-2,8	-1,3	-3,2	-2,2	-5,1	0,8		-1,0	-1,2		-2,2	PM0778
hexC	Capsule transport	-2,3	-2,6	-2,0	-3,8	-3,1	-4,8			-1,6	-1,7	-0,9	-1,8	PM0779
hexB	Capsule transport	-3,2	-3,0	-2,5	-3,9	-2,8	-4,8	0,6		-1,3	-1,4		-1,9	PM0780
hexA	Capsule transport	-2,1	-2,3	-1,7	-2,5	-1,7	-3,1			-1,0	-1,2	-0,7	-1,4	PM0781
wza	Capsule biosyn							2,1	2,7					PM1016
wzb	Capsule biosyn							2,6	3,4					PM1017
exbB	Iron transport	-2,5	-2,0	-2,3	-2,5	-2,0	-1,9	-1,1	-1,4	-1,5		-2,1	-1,3	PM1186
exbD	Iron transport	-1,5	-1,6	-2,3	-2,7	-2,1	-2,0	-0,8	-0,9	-1,5	-0,7	-1,8	-1,2	PM1187
tonB	Iron transport	-1,2	-1,3	-1,6	-1,6	-1,2	-1,3					-0,9	-1,2	PM1188
fbpB	Iron transport									-1,1	-0,9			PM0050
fbpA	Iron transport			-1,5	-1,3	-1,1	-0,7	0,6		-1,6		-1,2		PM0051
hugX	Iron metabolism					-1,6	-1,9			-1,0		-1,3	-0,8	PM0298
hugZ	Iron metabolism	-1,5		-2,2	-2,2	-1,7	-1,8			-1,6		-1,3		PM0299
hgbA	Iron transport								0,7					PM0300
fur	Iron regulation	0,7	0,7											PM0352
yfeA	Iron transport	-1,2				-0,6								PM0400
hemK	HemK protein						0,6							PM0556
afuA	Iron transport	-0,6		-0,7	-1,7	-0,9	-1,4							PM0954
afuB	Iron transport				-1,1		-1,5			-0,7	-1,1			PM0956
afuB	Iron transport								1,8	0,9	0,9			PM1455
afuC	Iron transport	-0,7			-1,0	-0,8	-1,5			-0,8				PM0957
PM1428	Iron transport							1,1	1,2					PM1428
hbpA	Iron transport				1,0					0,9		0,7		PM0592
tadF	Tight adherence							0,6	1,3					PM0844
tadE	Tight adherence							1,1	1,6					PM0845
tadD	Tight adherence	-1,5	-0,9					0,7	1,1					PM0846
tadC	Tight adherence							0,7	1,3					PM0847
tadB	Tight adherence	-1,5	-1,0		0,6			0,8	1,1					PM0848
tadA	Tight adherence							1,2	1,1	0,8	0,7			PM0849
lspB	Hemagglutinin	-2,2	-2,2			-1,3	-0,9			-0,7				PM0056
pfhB1	Hemagglutin. transp.	-2,8	-2,3	-1,3	-0,8	-1,4	-1,3							PM0057
pfhB2	Hemagglutinin	-1,3	-1,8	-0,9	-1,2	-1,3	-2,4			-0,7		-1,0	-1,2	PM0059
attK	Attachment protein							0,7		0,8	0,6			PM1536
PM0649	Hemolysin		0,6			-0,7	-0,9			0,7				PM0649
siaT	Sialyltransferase	1,8	1,4						-1,0					PM0188
comE	DNA competence	0,8		-1,3	-2,4		-2,3	-1,5	-1,9	-1,0	-1,2		-1,2	PM1225
comD	DNA competence			-1,2	-2,8		-3,1			-1,0	-1,1	-0,8	-1,5	PM1226
comC	DNA competence		-1,0		-2,8		-3,1	-2,3	-3,1	-0,9	-1,2			PM1227
comB	DNA competence	0,8	-0,9		-2,6		-2,4	-2,2	-2,9	-1,0	-1,2	-0,7	-1,5	PM1228
comA	DNA competence A		-1,2		-2,2		-2,0			-1,1	-1,4		-1,1	PM1229
comM	DNA competence	-5,0	-5,4		-1,2		-1,9		-1,4	-0,9				PM1510
comF	DNA competence	-1,4	-1,6											PM1556
galE	LPS biosynthesis	-1,4	-1,0	-0,6	-1,4		-1,2							PM0286
PM0507	LPS biosynthesis								1,0					PM0507
PM0508	LPS biosynthesis								0,8					PM0508
PM0509	LPS biosynthesis	0,7	0,8						0,9					PM0509
PM0512	LPS biosynthesis	0,8	0,7											PM0512
kdsA	LPS biosynthesis	1,0						1,0	0,8					PM0558
PM1294	LPS biosynthesis	0,6	0,8											PM1294
opsX	LPS biosynthesis			0,7										PM1302
kdtB	LPS biosynthesis	0,7	0,9											PM1304
kdsB	LPS biosynthesis					-0,7	-1,1		0,7					PM0858
rfaK	LPS biosynthesis		2,0	-1,5	-1,2	-1,1	-1,5							PM1144
PM1843	LPS biosynthesis	-1,3				-0,6	-0,8		0,7			0,6		PM1843
PM1863	LPS biosynthesis	-1,2	-0,7	0,6			0,8		0,8					PM1863
lpxB	LPS biosynthesis	-1,0					0,8							PM1997
glpQ	LPS modification	1,3	1,3		-1,2		-1,2				0,7			PM1444
PM1306	Glycosyl transf.			0,7	0,8	0,8					0,7			PM1306
nanU	Sialic acid metab.	-2,1			-0,9	-0,9	-1,3							PM1708
nanP	Sialic acid metab.	-0,8		-1,2	-1,9	-1,4	-2,2		0,7					PM1709

nanX	Sialic acid metab.						0,9	1,0											PM1710
nanE	Sialic acid metab.	-1,9					-0,7	-0,8											PM1711
nanK	Sialic acid metab.	-1,4					-0,7												PM1712
PM1713	Hypothetical	-1,5						-1,1	0,8	0,8									PM1713
PM1714	Regulator	-1,5							0,6	0,7									PM1714
PM0842	Regulator SirB-like	2,1	1,7																PM0842
nanA	Sialic acid metab.	-1,0							-1,1	0,9	0,9								PM1715
neuA	Sugar metabolism	1,9	1,3						-0,7									-0,6	PM0187
nagA	Sugar metabolism								-0,7	0,6	0,6								PM0874
nagB	Sugar metabolism								-1,1										PM0875
PM0876	Sugar metabolism									-0,7								-0,7	PM0876
oapA	Opacity protein								-0,6	0,8	1,3								PM0098
htrA	Protease								-1,2	-1,4	-1,6	-1,6							PM0734
degS	Protease									0,7	1,1							0,6	PM0748
clpP	Protease		0,6																PM1976
clpX	Protease		0,7															-0,7	PM1977
clpB	Protease		1,1	-1,1	-2,0	-1,8	-1,0	1,3										-0,9	PM1704
dam	DNA methylase	0,8	0,9	-0,7	-0,6													-0,6	PM1222
gcp	Sialoendopeptidase																	0,7	PM1238
cpxR	Reg. chemotaxis	-1,2																0,6	PM1887
cpxA	Sensor chemotaxis																	1,6	PM1888
oppD	Oligopept. transp.	-1,1																-0,6	PM1907
oppC	Oligopept. transp.	-1,2																-1,0	PM1908
oppB	Oligopept. transp.	-0,7																-0,9	PM1909
oppA	Oligopept. transp.		1,0	-0,7														-1,3	PM1910
sodC	Superox. dismutase																	0,9	PM1952
sodA	Superox. dismutase																	1,1	PM0001
hktE	Catalase	-1,3	0,8															1,2	PM0032
vacB	Virulence protein																	0,6	PM1954

* genes are arranged according to virulence functions. Log₂ values are presented.