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The LysR-type transcriptional regulator BsrA (PA2121) controls vital metabolic pathways in *Pseudomonas aeruginosa*

Magdalena Modrzejewska[1], Adam Kawalek[1], Aneta Agnieszka Bartosik[1]*

[1] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

* To whom correspondence should be addressed: anetab2@ibb.waw.pl

ABSTRACT

Pseudomonas aeruginosa, a facultative human pathogen causing nosocomial infections, has complex regulatory systems involving many transcriptional regulators. LTTR (LysR-Type Transcriptional Regulator) family proteins are involved in the regulation of various processes including stress responses, motility, virulence and amino acid metabolism. The aim of this study was to characterize the LysR-type protein BsrA (PA2121), previously described as a negative regulator of biofilm formation in *P. aeruginosa*. Genome wide identification of BsrA binding sites using ChIP-seq revealed 765 BsrA-bound regions in the *P. aeruginosa* PAO1161 genome, including 367 sites in intergenic regions. The motif T-N_{11}-A was identified within sequences bound by BsrA. Transcriptomic analysis showed altered expression of 157 genes in response to BsrA excess, of which 35 had a BsrA binding site within their promoter regions, suggesting a direct influence of BsrA on the transcription of these genes. BsrA-repressed loci included genes encoding proteins engaged in key metabolic pathways such as the tricarboxylic acid cycle. The panel of loci possibly directly activated by BsrA, included genes involved in pili/fimbriae assembly as well as secretion and transport systems. In addition, DNA pull-down and regulatory

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24 analyses showed the involvement of PA2551, PA3398 and PA5189 in regulation of *bsrA*
25 expression, indicating that this gene is part of an intricate regulatory network. Taken together,
26 these findings reveal the existence of a BsrA regulon, which performs important functions in *P.*
27 *aeruginosa*.

28

29 **IMPORTANCE**

30 This study shows that BsrA, a LysR-type transcriptional regulator from *P. aeruginosa*,
31 previously identified as a repressor of biofilm synthesis, is part of an intricate global regulatory
32 network. BsrA acts directly and/or indirectly as the repressor and/or activator of genes from vital
33 metabolic pathways (e.g. pyruvate, acetate, tricarboxylic acid cycle), and is involved in control
34 of transport functions and the formation of surface appendages. Expression of the *bsrA* gene is
35 increased in the presence of antibiotics, which suggests its induction in response to stress,
36 possibly reflecting the need to redirect metabolism under stressful conditions. This is particularly
37 relevant for the treatment of infections caused by *P. aeruginosa*. In summary, the findings of this
38 study demonstrate that the BsrA regulator performs important roles in carbon metabolism,
39 biofilm formation and antibiotic resistance in *P. aeruginosa*.

40

41 **KEYWORDS:** *Pseudomonas aeruginosa*, LysR-type transcriptional regulator (LTTR), BsrA
42 regulon, tricarboxylic acid cycle, regulatory network

43

44 **INTRODUCTION**

45 Regulation of transcription is the principal mechanism controlling gene expression and
46 the most economical way for a cell to respond to a rapidly changing environment. One of the

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47 largest groups of transcriptional regulators, with representatives in bacteria, archaea, and even
48 eukaryotic organisms (1, 2), is the LysR Type Transcriptional Regulator (LTTR) family (3).
49 Most LTTRs have two conserved and similarly organized functional domains (1, 4). The N-
50 terminal DNA binding domain (DBD) with a winged helix-turn-helix motif mediates binding to
51 cognate promoter sequences. The C-terminal effector binding domain (EBD), usually composed
52 of two response subdomains (RD1 and RD2), is involved in ligand recognition and modulation
53 of DBD activity (1, 3, 5). The conserved subdomain RD1 is also important for DNA interactions,
54 whereas the more diverse RD2 contains an effector binding site (1, 6). LTTRs mediate signal-
55 dependent and signal-independent transcriptional regulation of genes involved in numerous
56 cellular processes, such as oxidative stress response, cell wall shape determination, quorum
57 sensing, regulation of efflux pumps, secretion, motility, nitrogen fixation, virulence, cell
58 division, metabolism and recognition of environmental stimuli and stresses (1).

59 The targets of LTTR regulation are often transcribed from a promoter that is very close to
60 and may overlap that of a divergently transcribed regulator gene. In many cases, the LTTR
61 positively regulates the target promoter in an effector-responsive manner, while negatively
62 autoregulating its own promoter in the absence of an inducer (7–11). LTTRs can bind to target
63 promoters in two conformations, depending on the presence of an effector. Ligand binding by the
64 LTTR triggers a conformational change that permits binding to a DNA sequence involved in the
65 regulation of its target gene. LTTRs may act as multimers, most frequently tetramers (12).
66 Studies on several LTTRs have shown that the apoproteins can bind their promoters as tetramers,
67 causing an extended DNase I footprint and a high-angle DNA bend, while the corresponding
68 holoproteins produce a smaller footprint and lower DNA bend angle (1, 5). LTTRs usually bind
69 to a sequence of approximately 50–60 bps, containing two distinct sites: a recognition-binding

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70 site or repression-binding site (RBS), encompassing the sequence T-N_{11}-A (LTTR box), often
71 located around position -65 relative to the start of transcription, and an activation-binding site
72 (ABS) consisting of the -35 (ABS-35) and -10 (ABS-10) promoter regions (1, 5). In the absence
73 of inducer, the LTTR tetramer binds to a RBS, but also with low affinity to the ABS-10 site,
74 causing a bend in the DNA, leading to repression of the target gene by blocking availability of
75 the -35 promoter region (13–15). The bent DNA is relaxed upon effector binding to the LTTR,
76 leading to the formation of an active complex with RNA polymerase to initiate transcription. A
77 'sliding dimer' mechanism was proposed in which activation of the LTTR leads to a shift in the
78 binding site from RBS/ABS-10 to RBS/ABS-35, releasing the -35 box for RNA polymerase
79 recognition and subsequent gene expression (16, 17). Concomitantly, the autoregulatory
80 properties of LTTRs are thought to be connected only with the dimeric form of the protein, not
81 bound to the effector. The LTTR might bind to the RBS region of its own gene in a ligand-
82 independent manner to regulate its expression (1).

83 One of the largest repertoires of LTTRs is encoded in the genome of *Pseudomonas*
84 *aeruginosa*, an opportunistic human pathogen causing nosocomial infections including
85 septicaemia, urinary tract infections, pneumonia, skin and wound infections (18–22). About 10%
86 of all *P. aeruginosa* genes (usually around 6000) encode transcription factors. In the first
87 sequenced *P. aeruginosa* genome of reference strain PAO1 (23), 113 genes are annotated as
88 encoding LysR-type transcriptional regulators, but their functions remain largely unknown. *P.*
89 *aeruginosa* LTTRs with known roles include PA0133 (BauR) (24), PA0739 (SdsB1) (25),
90 PA1413 (26), PA1422 (GbuR) (27), PA1998 (DhcR) (28), PA2076 (OdsR) (29), PA2206 (30),
91 PA2258 (PtxR) (31), PA2432 (BexR) (32), PA2838 (33), PA3225 (34), PA3587 (MetR) (35),
92 PA3630 (GfnR) (36), PA4109 (AmpR) (37), PA4203 (38), PA5437 (PycR) (39), PA1003

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93 (MvfR, also called PqsR) (40–42), PA5344 (OxyR) (43–45) and PA2492 (MexT) (46, 47). The
94 membrane-associated multiple virulence factor regulator MvfR was shown to be necessary for *P.*
95 *aeruginosa* virulence (40). MvfR positively regulates production of the *Pseudomonas* quinolone
96 signal (PQS), one of three *P. aeruginosa* quorum sensing systems (48, 49), by controlling the
97 *pqsABCDE* operon (50), as well as the *phnAB* genes involved in the biosynthesis of phenazine
98 and anthranilic acid, a precursor of PQS (50, 51). Recent reports indicate that MvfR binds to
99 dozens of loci across the *P. aeruginosa* genome at promoter regions, and within and outside the
100 coding sequences of genes, recognizing different DNA binding motifs (41, 42), suggesting its
101 involvement in the regulation of multiple genes. OxyR, another well characterized *P. aeruginosa*
102 LTTR, is involved in the oxidative stress response, acting as a redox sensor (43). OxyR is
103 activated by hydrogen peroxide (H_2O_2) and protects cells from toxic oxygen derivatives by
104 stimulating the expression of the *katA*, *katB*, *ahpB* and *ahpCF* genes encoding catalases and alkyl
105 hydroperoxide reductases (43, 52). It was recently shown that OxyR also regulates several other
106 processes such as iron homeostasis, pyocyanin production and quorum sensing by binding to an
107 AT rich motif (44, 45, 53). Another example of a *P. aeruginosa* LTTR with multiple roles is
108 MexT (PA2492), an activator of the *mexEF-oprN* operon encoding a multidrug efflux pump
109 involved in resistance to quinolones, chloramphenicol, trimethoprim and imipenem (46, 47, 54).
110 Besides this handful of well-studied examples, the majority of LTTRs in this important pathogen
111 remain uncharacterized.

112 Recently, a putative LTTR PA2121 was shown to negatively affect biofilm synthesis in
113 the *P. aeruginosa* strain PAK and was therefore named biofilm synthesis repressor BsrA (55). It
114 was shown, that the *bsrA* gene is regulated by the small regulatory protein SrpA during phage
115 infection (56). SrpA is a key regulator controlling core cellular processes in *P. aeruginosa* PAK,

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116 including biofilm formation, and this factor binds to the motif TATC-N9-GATA identified
117 within the *bsrA* promoter region.

118 In this study, we analyzed the role of BsrA in *P. aeruginosa* strain PAO1161, a derivative
119 of PAO1 (57). In contrast to PAK, neither of these strains encodes *srpA* homologues. Our data
120 indicate that the mode of BsrA action may differ in the strains PAK and PAO1161, because
121 under the conditions tested, BsrA deficiency or overproduction had no influence on biofilm
122 formation in PAO1161. Using RNA sequencing and chromatin immunoprecipitation we
123 identified a BsrA regulon, which encompasses a gene encoding a key enzyme of the
124 tricarboxylic acid cycle (TCA), a small RNA, as well as genes engaged in different cellular
125 processes, some that are potentially involved in biofilm production. Using a DNA pull-down
126 assay and regulatory experiments, we showed that other LysR-type regulators bind and regulate
127 the *bsrA* promoter. Thus, BsrA is a part of an intricate regulatory network, that controls
128 metabolic pathways during adaptation to a changing environment.

129

130 **RESULTS**

131 **Impact of *bsrA* deficiency or overexpression on bacterial physiology**

132 To analyze the role of BsrA in *P. aeruginosa*, a PAO1161 Δ*bsrA* mutant was constructed.
133 This mutant strain did not display any significant differences in growth in LB or M9 medium,
134 colony morphology, swimming or swarming, compared to the wild type (WT) parental strain
135 PAO1161 (Fig. S1ABC). In parallel, the effect of *bsrA* overexpression was tested by linking the
136 gene to an IPTG-inducible promoter in plasmid pMEB63 (*lacI*^{Q}-*tac*p-*bsrA*). No effects of BsrA
137 overproduction on bacterial growth were observed when IPTG concentrations of ≤ 0.25 mM

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138 were used (Fig. S1D), whereas 0.5 mM IPTG reduced the rate of growth significantly compared
139 to cells carrying the empty vector (Fig. S1D).

140 As *bsrA* was initially identified as a repressor of biofilm synthesis, the formation of
141 biofilms by the strains lacking or overproducing BsrA was examined. The absence of *bsrA* had
142 no effect on the production of a biofilm by cells grown in either LB or M9 medium (Fig. 1A).
143 Furthermore, the addition of arginine or a sub-inhibitory concentration of streptomycin to the
144 growth medium, two compounds known to promote biofilm synthesis in *P. aeruginosa* (58, 59),
145 resulted in comparable increases in biofilm formation in WT and Δ*bsrA* cells (Fig. 1A).
146 Similarly, an excess of BsrA did not affect biofilm formation (Fig. 1B). These data suggested
147 that BsrA may play an auxiliary or strain-specific role in biofilm formation in *P. aeruginosa*.

148 **Identification of BsrA-regulated genes and binding sites for this transcriptional regulator**
149 **in the *P. aeruginosa* genome**

150 To identify genes that display BsrA-dependent expression we used RNA sequencing
151 analysis (RNA-seq) to characterize the transcriptome of *bsrA*-overexpressing cells. In addition,
152 we performed chromatin immunoprecipitation and sequencing analysis (ChIP-seq) to identify
153 BsrA binding sites in the *P. aeruginosa* genome. The rationale behind an analysis of cells with
154 BsrA in excess rather than the Δ*bsrA* mutant, was based on the following: 1) the relatively low
155 level of *bsrA* expression under standard growth conditions (LB or M9 medium, data not shown);
156 2) the likelihood that an excess of BsrA might mimic the induced, activated state of the protein,
157 and 3) the fact that the effector for this LTTR is unknown.

158 RNA-seq was performed using material isolated from cultures of the strains PAO1161
159 pMEB63 (*lacI*^{Q}-*tac*p-*bsrA*, hereafter called BsrA+) and PAO1161 pAMB9.37 (*lacI*^{Q}-*tac*p, empty
160 vector - EV) grown in selective LB supplemented with 0.05 mM IPTG (Data set S1).

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161 Comparison of the BsrA+ and EV transcriptomes identified 157 loci with altered expression
162 [fold change (FC) $\leq$ -2 or $\geq$ 2, FDR adjusted *p*-value $\leq$ 0.01] (Fig. 2A; Data set S2). The
163 expression of 65 loci was down-regulated, while 92 loci displayed increased expression. For
164 convenience, we use the *P. aeruginosa* PAO1 gene names throughout the manuscript, although
165 the corresponding PAO1161 gene names are included in all tables. Functional classification of
166 the identified loci, based on PseudoCAP (60), showed that the up-regulated genes were mostly
167 involved in protein secretion/export systems, adaptation and protection as well as cell wall
168 functions (Fig. 2B; Data set S2). Decreased expression was observed for several genes encoding
169 proteins engaged in carbon compound metabolism and central intermediary metabolism. The
170 most severely down-regulated genes were *PA3452* (*mqoA*), encoding a malate:quinone
171 oxidoreductase from the TCA cycle and *PA0887* (*acsA*) encoding an acetyl-coenzyme A
172 synthetase (61, 62), while the most highly up-regulated loci were the *mexXY* operon, encoding a
173 multidrug efflux RND transporter (63–65), as well as genes encoding type VI secretion proteins
174 (*PA1657-PA1671*) and transporters (*PA4192-PA4195*, *PA2202*, *PA2203*, *PA5024*) (Data set S2).
175 The altered expression of selected loci in response to BsrA excess was confirmed using RT-
176 qPCR analysis (data not shown).

177 To identify BsrA binding sites in the *P. aeruginosa* genome, ChIP-seq analysis was
178 performed using an anti-FLAG antibody and Δ*bsrA* cells carrying plasmid pMEB99 (*tac*p-*bsrA*-
179 *flag*), grown in selective LB supplemented with 0.05 mM IPTG. Addition of a FLAG-tag to the
180 C-terminus of BsrA did not alter its ability to retard bacterial growth when overproduced (Fig.
181 S2), indicating that the fusion protein is functional. As a background control for the ChIP
182 procedure, the Δ*bsrA* strain carrying plasmid pABB28.1 (*tac*p-*flag*) was grown under the same
183 conditions and samples were processed in parallel. Comparison of BsrA-FLAG ChIP samples

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184 with control samples, using a fold enrichment (FE) cut-off value of 2 (Fig. 2C) yielded 765
185 BsrA-FLAG ChIP-seq peaks (Data set S3). The majority of peaks exhibited an FE of between 2
186 and 4, although 166 had FE values of 4 to 10, and 21 had an FE of > 10 (Fig. 2C). The mean
187 width of ChIP-seq peaks was < 1000 (twice the length of the DNA fragments used for ChIP),
188 indicating BsrA binding to single or closely spaced binding site(s) (Fig. 2D). The summits of 367
189 peaks (48%) mapped to intergenic regions (Data set S3). A similar analysis of peak summit
190 positions relative to the start codons of PAO1161 open reading frames (or the first genes in
191 operons) showed that 426 peaks were located in the -500 to +100 regions, which suggests that
192 the expression of these loci could be regulated by BsrA (Fig. 2E).

193 An extensive search for nucleotide motifs shared by sites bound by BsrA using MEME
194 (66), showed the presence of a consensus sequence, resembling the T-N_{11}-A motif (LTTR box)
195 (Fig. 2F) proposed as the binding site of other LTTRs (1, 67, 68). These data indicated that BsrA
196 has multiple binding sites in the *P. aeruginosa* genome, which suggests that this factor may
197 function as a modulator of gene expression in regulatory networks.

198 **Genes under the direct control of BsrA**

199 Interestingly, 35 of the 157 genes showing altered expression in response to a BsrA
200 excess possessed a binding site for this transcriptional regulator within their promoter regions
201 (Fig. 2G, Fig. 3ABC, Table 1). In addition, 55 BsrA peaks detected in coding regions were in the
202 vicinity of genes that showed changes in expression level (fold change > 1.5) in RNA-seq
203 analysis (Data set S3), but the mechanism by which BsrA could influence their expression
204 requires further studies.

205 Our analysis confirmed that BsrA might bind within the region preceding its own coding
206 sequence (Fig. 3A). A BsrA binding site was also detected in the putative promoter of *PA3452*

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207 (*mqoA*): the gene showing the most severe down-regulation in the RNA-seq analysis (FC= -3.86)
208 (Fig. 3B). Among the genes that might be directly regulated by BsrA, *PA1112.1*, encoding a
209 small non-coding RNA (ncRNA) of unknown function (69), had a peak with the greatest fold
210 enrichment (12.7) in the region preceding the structural gene (Fig. 3C).

211 To confirm the interactions of BsrA with putative promoters of these genes, we
212 performed electrophoretic mobility shift assays (EMSA) using purified His_6-BsrA and DNA
213 fragments corresponding to the putative promoter regions of *bsrA*, *PA3452* and *PA1112.1*. Shifts
214 of the promoter fragment DNA bands, but not of a non-specific competitor DNA were observed,
215 indicating that His_6-BsrA binds to these regions *in vitro* (Fig. 3DEF). To verify the importance of
216 the LTTR box sequences in DNA binding by BsrA, version of the *PA1112.1* promoter fragment
217 lacking the T-N_{11}-A motif was tested in an EMSA. No BsrA binding to this shortened fragment
218 (232 bp instead of 303 bp) could be detected (Fig. 3G).

219 To further examine the influence of BsrA on the expression of the three aforementioned
220 genes, their promoter regions were cloned upstream of a promoter-less *xylE* gene in the vector
221 pPTOI. The *bsrA* and *PA1112.1* promoters were active in the heterologous host *E. coli* DH5α,
222 whereas no activity was observed for *PA3452*p (Fig. 3HI and data not shown). Expression of
223 BsrA in cells carrying plasmids with *bsrA*p-*xylE* or *PA1112.1*p-*xylE* resulted in significantly
224 reduced XylE activity in the corresponding cell extracts (Fig. 3HI). Moreover, RT-qPCR
225 analysis of *PA3452* (*mqoA*) and *PA1112.1* transcript levels in *bsrA*-deficient cells showed
226 increased expression of these two genes relative to WT cells, which supported the repressive
227 effect of BsrA on the transcription of these genes (Fig. 3JK).

228 These data confirmed that BsrA binds to DNA fragments identified in ChIP-seq analysis
229 and may regulate the activity of target promoters to influence gene expression. In addition, the T-

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230 N_{11}-A nucleotide sequence, known as the LTTR box, present in the binding sites of most LTTRs
231 (1, 67), is recognized by BsrA.

232 **Modulation of different cellular processes by BsrA**

233 The RNA-seq results suggested that BsrA is engaged in modulating the activity of
234 proteins mediating the conversion of malate to oxaloacetate in the TCA cycle by repressing the
235 expression of the *PA3452* (*mqoA*) and *PA4640* (*mqoB*) genes (Data set S1). This is likely to
236 influence subsequent steps of the cycle, e.g. the availability of oxaloacetate, its conversion to
237 citrate using acetyl-CoA or the levels of acetyl-CoA generated via the pyruvate shunt (Fig. 4A).
238 In addition, several genes that are putatively involved in the acetate transport (*PA3233*, *PA3234*)
239 (70) and acetate pathways [*acsA* (*PA1562*), *acsB* (*PA1787*), *exaC* (*PA1984*)], encoding probable
240 succinyl-CoA/acetate CoA-transferase (*PA5445*) (71), also showed reduced expression (FC
241 >1.5) in response to BsrA (Fig. 4A; Table S1) (72, 73), suggesting the involvement of this LTTR
242 in controlling acetate metabolism. We cultured the WT and Δ*bsrA* strains in minimal medium
243 supplemented with citrate or acetate as the sole carbon source, but no visible effects on the
244 kinetics of growth were observed (Fig. S1B). To test the effect of BsrA on acetate metabolism,
245 the two strains were also cultured in medium containing a sub-inhibitory concentration of
246 kanamycin, following the report of Meylan and co-workers, showing the effect of central carbon
247 metabolite stimulation on aminoglycoside sensitivity in *P. aeruginosa* (74). The propagation of
248 cells from overnight cultures in M9 medium containing 50 μg/mL kanamycin and acetate as the
249 sole carbon source resulted in an increase in cfu/ml (relative to the starting point) of the *bsrA*-
250 deficient mutant, while the cfu/ml value of the WT strain was not significantly changed (Fig.
251 4B). This effect was not observed when pyruvate and fumarate (compounds from different parts
252 of the TCA cycle), or acetate plus fumarate, were used as the carbon source(s). Thus, the *P.*

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253 *aeruginosa* Δ*bsrA* mutant exhibited higher survival and/or fitness than the WT strain in the
254 presence of kanamycin when grown in minimal medium supplemented with acetate as the sole
255 carbon source, which confirmed the influence of BsrA on acetate metabolism.

256 To test the effect of various antibiotics on *bsrA* expression, we performed RT-qPCR
257 using RNA isolated from PAO1161 cultures grown in medium supplemented with sub-inhibitory
258 concentrations of different antibiotics. This analysis showed no significant difference in *bsrA*
259 expression upon the addition of kanamycin or ciprofloxacin compared with a negative control
260 culture (Fig. 4C). Interestingly, the expression of *bsrA* was significantly increased in response to
261 spectinomycin, streptomycin, tetracycline and carbenicillin (Fig. 4C), which indicates that *bsrA*
262 is induced in response to specific antibiotics.

263 Our RNA-seq and ChIP-seq results also indicated increased expression of genes involved
264 in fimbriae assembly (e.g. *PA0499*, *PA4648*-*PA4653*) in response to BsrA in excess. PA0499 is a
265 periplasmic protein predicted to act as a chaperone assisting the assembly of appendages on the
266 surface of the bacterium (75). *PA4648* is the first gene of the six-component *cupE* cluster
267 encoding a so-called chaperone-usher pathway, the activation of which leads to the production
268 and assembly of CupE fimbriae on the cell surface (76). These fimbriae are known to play a
269 crucial role in biofilm development by *P. aeruginosa* and the *cupE* operon is specifically
270 expressed in biofilm-forming cells (76). Since biofilm formation was unaffected in both the
271 Δ*bsrA* and BsrA+ strains (Fig. 1), we checked whether *bsrA* overexpression had any effect on
272 swimming, twitching or swarming motilities (77–79). BsrA+ cells showed differences in
273 twitching (involves pili) and swarming, as demonstrated by the presence of clear radiating
274 motility zones (“lines”) spreading from the centre of bacterial colonies, that were not observed in
275 the control strain. This may reflect possible changes in radial expansion of the colony, which

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276 could be related to enhanced appendage production in BsrA+ cells (Fig. 4DE). No effect on
277 swimming was observed (Fig. 4F), indicating that BsrA overproduction does not have a general
278 negative effect on the motility of cells grown on plates. Thus, BsrA appears to be involved in the
279 regulation of swarming and twitching motilities, and possibly attachment to surfaces, the first
280 stage in biofilm formation.

281 Taken together, these results demonstrated the participation of BsrA in a number of
282 diverse cellular processes including the modulation of cellular metabolism in response to growth
283 conditions and the control of appendage formation leading to altered motility of *P. aeruginosa*
284 cells.

285 **BsrA is under the control of other transcriptional regulators in *P. aeruginosa***

286 The findings of a previous study (55) and our data showed that the expression of *bsrA* is
287 subject to autoregulation. To identify other proteins that can modulate *bsrA* transcription and in
288 consequence the level of BsrA, we used a *bsrA* promoter fragment as bait in a DNA pull-down
289 assay with *P. aeruginosa* PAO1161 cell extracts. The proteins bound to *bsrA*p were then
290 characterized by mass spectrometry analysis. Altogether, 39 proteins were identified as being
291 able to bind to *bsrA*p, but not to a control DNA fragment Data set S4). Importantly, BsrA was
292 identified among the proteins with the highest scores, providing a positive control for this
293 approach and confirming the autoregulatory properties of the protein. Six other proteins were
294 identified with high scores for binding to *bsrA*p in two independently tested samples (eluates):
295 PA2551, PA3587 (MetR), PA4902, PA4462 (RpoN), PA5189 and PA3398. Interestingly, five of
296 these proteins are classified as LysR family transcriptional regulators, whereas PA4462 (RpoN)
297 is a σ^{54} factor interacting with RNA polymerase (80). It is known that σ^{54} factors direct RNAP to

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298 conserved -12 (TGC) and -24 (GG) elements, and similar regions (TGA at position -12 and GG
299 at position -24) are present in the *bsrA*p.

300 To determine whether the proteins identified in pull-down analysis can indeed affect the
301 activity of the *bsrA* promoter, the *PA2551*, *PA3398*, *PA3587*, *PA4902* and *PA5189* genes were
302 cloned under the control of *tac*p in vector pAMB9.37 and expressed in cells carrying plasmid
303 pMEB190 (*bsrA*p-*xylE*). Measurements of XylE activity in cell extracts of the double
304 transformants showed that the expression of *PA3587* and *PA4902* did not significantly influence
305 *bsrA*p activity under the tested conditions (Fig. 5A). Notably expression of *PA2551*, *PA3398* or
306 *PA5189* resulted in major decreases in XylE activity, suggesting that these proteins act directly
307 as repressors of the *bsrA* gene. Interestingly, ChIP-seq analysis revealed strong binding of BsrA
308 upstream of the *PA2551*, *PA3398* and *PA5189* genes (Fig. 5B), but not *PA3587* or *PA4902* (data
309 not shown).

310 These results showed that BsrA is part of an intricate regulatory network involving
311 mutual regulation between BsrA and other LysR-type transcription factors.

312

313 **DISCUSSION**

314 In this study, we performed a functional analysis of the LysR-type transcriptional
315 regulator BsrA (PA2121) from *P. aeruginosa*, previously described as a repressor of biofilm
316 synthesis (55).

317 Transcriptional analysis of a strain overproducing BsrA revealed the greatest changes in
318 gene expression for loci encoding enzymes engaged in carbon metabolism (mainly down-
319 regulated) and for loci predicted or known to be involved in processes connected with transport,
320 biofilm and type VI secretion systems (up-regulated). In a *P. aeruginosa* PAK mutant with

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321 disrupted *bsrA*, increased biofilm synthesis was observed (55), while the PAO1161 Δ*bsrA*
322 mutant constructed in this study did not show significant changes in biofilm formation (Fig. 1).
323 This difference could be related to the presence of the SrpA protein in the PAK strain, which is
324 not encoded in the genome of PAO1161 (or PAO1). Among its other functions, SrpA directly
325 regulates expression of the *bsrA* gene by binding to its promoter (56). Our data suggested that
326 another mechanism is responsible for regulating biofilm production, possibly involving BsrA-
327 mediated activation of genes such as *PA0499* or *PA4648*, that have been connected with the
328 formation of biofilms (75, 76). The generation of biofilm structures is strictly linked to metabolic
329 activity, inhibited in the cells of the mature biofilm matrix, but increased during early biofilm
330 development (81). The role of BsrA in biofilm formation might be related to the modulation of
331 these processes. The relationship between SrpA and BsrA in biofilm formation requires further
332 study.

333 Our data suggested that BsrA is involved in the repression of metabolic functions by
334 direct or indirect down-regulation of genes engaged in pyruvate metabolism and the TCA cycle
335 (Fig. 4A; Fig. 6). The most highly repressed gene, directly controlled by BsrA, is *mqoA*
336 (*PA3452*) encoding a putative malate:quinone oxidoreductase (MQO), a FAD-dependent enzyme
337 involved in the conversion of malate to oxaloacetate. The gene encoding the second *P.*
338 *aeruginosa* MQO, *mqoB* (*PA4640*), was also subject to BsrA-mediated regulation, although to a
339 lesser extent. The presence of *mqoB* is necessary for the growth of cells on acetate and ethanol as
340 sole carbon sources (82). Under these conditions, one of the primary functions of MQOs is to
341 replenish the oxaloacetate pool in the TCA cycle to allow further assimilation of acetyl-CoA and
342 permit TCA operation to provide intermediates for biosynthetic processes and respiration (82).
343 Both MQOs are produced by cells grown under standard aerobic conditions, but levels of MqoB

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344 are higher than those of MqoA (33, 73, 82). The precise role of MqoA in *P. aeruginosa* awaits
345 elucidation. Bacterial MQOs have previously been characterized in *E. coli* and *Corynebacterium*
346 *glutamicum* as the principal enzymes catalysing the oxidation of malate (61, 83). In
347 *Pseudomonas putida* the *mqo-2* gene, encoding a malate:quinone oxidoreductase 2, is under the
348 control of Crc, the global regulator of carbon catabolite repression (CCR) (84). The assimilation
349 of energetically favourable carbon sources is the main bacterial strategy employed to optimize
350 metabolism and growth. Crc protein together with the RNA chaperone translational repressor
351 Hfq and small RNA(s) comprise the CCR regulatory system in pseudomonads (85, 86). In *P.*
352 *aeruginosa*, a specific sRNA named CrcZ has been identified as an antagonist of Crc and Hfq.
353 CrcZ binds to the Crc and Hfq proteins, trapping and sequestering them. The expression of *crcZ*
354 is under the control of a two-component system CbrA/CbrB, which reacts to carbon source
355 availability (85, 87, 88). It is clear that a multilevel regulatory network involving sRNAs plays
356 an important role in metabolic regulation in pseudomonads, which is interesting in light of our
357 identification of a sRNA (*PA1112.1*) as a target of BsrA regulation.

358 Our analysis of the phenotype of the *bsrA*-deficient mutant demonstrated its increased
359 fitness in the presence of kanamycin compared to the WT strain under specific conditions. It was
360 previously recognized that the efficacy of aminoglycoside antibiotics depends on metabolic
361 stimuli (74, 89, 90). As an aminoglycoside, kanamycin acts by inhibiting protein synthesis
362 through binding to the 30S subunit of the bacterial ribosome. Killing of bacterial cells by
363 kanamycin depends on proton-motive force (PMF), which is required for the uptake of the
364 antibiotic (89). PMF is related to NADH level, which is dependent on metabolism. Therefore, the
365 cellular metabolic state modulates the uptake and/or efficacy of the antibiotic (90). Although
366 adaptation to antibiotics is thought to be controlled at the transcriptional level by the induction of

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367 stress responses, several reports have indicated that there is a relationship between a high
368 concentration of certain endogenous metabolites and the level of bacterial resistance (91–94).
369 We found that the Δ*bsrA* mutant displayed better adaptation to kanamycin under conditions of
370 acetate supplementation and it may be speculated that this is due to altered drug uptake due to
371 changes in PMF generation, a process connected with the TCA cycle and cellular respiration (89,
372 90, 95). Growth on acetate requires the activity of the glyoxylate shunt which supplies cells with
373 malate and oxaloacetate (Fig. 4A). It might be connected with reoxidization of the NADH excess
374 generated by the TCA cycle during growth on acetate and the need to coordinate the composition
375 of the electron transport chain at the level of the terminal oxidases, e.g. the proton pumping
376 NADH dehydrogenase I or Nqr (73). The Δ*bsrA* mutant had an increased level of the transcript
377 of malate dehydrogenase *mqoA* (Fig. 3J) and probably those encoding several other enzymes
378 from the TCA cycle and acetate metabolism. The lack of repression of TCA cycle enzymes or
379 genes involved in acetate metabolism in the Δ*bsrA* mutant in comparison with the WT strain may
380 provide some advantage during growth on acetate in the presence of kanamycin and adaptation
381 to the stress caused by this antibiotic.

382 Kanamycin sensitivity was examined in cells grown on other carbon sources, but a
383 significant difference in antibiotic adaptation of the Δ*bsrA* mutant was only observed with
384 acetate supplementation. The main reason for this may be the stage at which particular carbon
385 compounds enter the TCA cycle, as shown by Dolan and co-workers (73). These authors
386 presented so-called "carbon fluxes" leading to metabolic and transcriptomic changes caused by
387 growth on acetate or glycerol. We speculate that the lack of BsrA leads to elevated TCA cycle
388 flux connected with metabolism remodelling when acetate is the sole carbon source.

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389 An interesting gene belonging to the BsrA regulon, potentially connected with TCA cycle
390 remodelling, is *PA5445.* This gene putatively encodes succinyl-CoA/acetate CoA-transferase, an
391 enzyme engaged in the conversion of succinyl-CoA and acetate to succinate and acetyl-CoA,
392 which could modulate the TCA cycle and confer some advantage during growth on acetate.
393 PA5445 displays almost 50% identity to AarC from *Acetobacter aceti*, a bacterium utilizing a
394 specialized TCA cycle (71). In this bacterium AarC-mediated conversion of succinyl-CoA to
395 succinate replaces the action of typical succinyl-CoA synthetases (SucC, SucD) (71, 96). This
396 modification is connected with enhanced tolerance to low pH and acetate, produced by
397 *Acetobacte*r during fermentation. Many bacteria including *P. aeruginosa* possess homologues of
398 *aarC* (*asct*) in addition to the *sucC* and *sucD* genes, which suggests the existence of an
399 alternative pathway in the TCA cycle, possibly conferring some advantage connected with
400 acetate metabolism (96).

401 Similarly to *mqoA*p, the promoter region of *PA5445* possesses few potential BsrA
402 binding sites (matching the consensus in Fig. 2F), with one putative site (TTCGACCTTGGTA)
403 overlapping the predicted -10 promoter region and located very close to a BsrA ChIP-seq peak
404 summit. This suggests that BsrA may regulate genes encoding components of metabolic
405 pathways and can mediate metabolism remodelling, which could lead to increased fitness of the
406 Δ*bsrA* mutant in the presence of kanamycin.

407 Interestingly, *bsrA* (*PA2121*) was identified as one of a panel of genes containing
408 mutations in *P. aeruginosa* cystic fibrosis isolates, which may have been selected during
409 adaptation and evolution to promote survival during infection of the lungs of these patients (97–
410 99). In addition, Kong and co-workers (100), using a *luxCDABE*-based random promoter library

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411 of *P. aeruginosa* PAO1, identified *PA2121* (*bsrA*) as one of 45 genes that perform a role in long-
412 term survival and thus may be involved in chronic infections of the human body.

413 BsrA binds to numerous sites in the *P. aeruginosa* genome, yet it only had a limited
414 influence on the regulation of gene expression under the conditions tested (Data set S3). The
415 majority of BsrA binding sites contain the LTTR box, composed of the sequence T-N_{11}-A, but
416 besides this element there is a low level of sequence conservation. It was not possible to define a
417 more specific binding motif, which suggests the involvement of other factors in mediating BsrA
418 binding to DNA. This observation highlights the potentially broad role of BsrA in modulating
419 gene expression in *P. aeruginosa*, with the possible involvement of other regulatory proteins that
420 associate with sequences adjacent to BsrA binding sites under specific growth conditions. The
421 nature of the signal to which BsrA responds and the precise role of this factor, require further
422 study.

423 Recently, high-throughput SELEX analysis has been used to define the preferred binding
424 motifs of 53 *P. aeruginosa* LysR-type transcriptional regulators (101). Most of these LTTRs
425 display dimeric binding to cognate sequences. The recognised binding sites are mostly
426 palindromic or have partial dyad symmetry and range in length from 12 to 24 base pairs.
427 Sequence conservation is highest within the flanking regions, that usually display dyad
428 symmetry, whereas there is often very low sequence conservation inside the motif. In most of the
429 binding sites the LTTR-box (T-N11-A, T-N10-A or T-N9-A) can be identified as part of the
430 sequence creating dyad symmetry. The motif preferentially recognized by BsrA was identified as
431 NAGTAGACNNGTCTACTN; however, no such sequence was found in the genomes of PAO1
432 or PAO1161 and no highly similar sequences were present in the regions identified using ChIP-
433 seq analysis. FIMO analysis (102) using 200-bp sequences encompassing the BsrA peak

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434 summits identified only 5 sequences with a *p*-value of < 0.0001 resembling the proposed motif
435 [peaks 682, 194, 367, 276, 157 (Data set S3)] or 56 sequences when a *p*-value cut-off of 0.001
436 was used. The preferential BsrA binding site motif identified in our analysis is more generic, but
437 is recognizable as an LTTR box characteristic for LysR-type regulators, and better explains the
438 presence of multiple LTTR binding sites within the promoters of cognate genes.

439 LTTRs usually bind to promoters of target genes upstream from the transcription start
440 site. Among the tested promoter regions of BsrA regulated genes, i.e. *bsrA*, *mqoA* and *PA1112.1*,
441 two to four T-N_{11}-A motifs, closely resembling the BsrA binding site (Fig. 2F) were identified
442 (Fig. 3A-C). These are located at positions from 3 to 184 bp from the start codon of these down-
443 regulated gene, so that BsrA binding to these sites might reduce RNA polymerase access to the
444 core promoter sequences (-10, -35). To specifically recognize and bind cognate DNA, LTTRs
445 use highly conserved interactions between amino acids and nucleotide bases as well as numerous
446 less conserved secondary interactions (7, 68). One site, often called the recognition binding site,
447 consists of a T-N_{11}-A motif with imperfect dyad symmetry. It is believed that interaction with
448 this site anchors the LTTR to the DNA and is often involved in repression, including
449 autoregulation. LTTRs are known to bind to longer sequences (50-60 bps) containing a so-called
450 activation binding site, and these interactions are usually driven by the presence of a specific
451 ligand or co-factor, which is bound by the LTTR. In addition, LTTRs bind with higher or lower
452 affinity to their binding sites depending on the presence or absence of its inducer or ligand,
453 which modulates interaction with DNA. Conformational flexibility of the created LTTR
454 multimers (usually tetramers), causes DNA bending or relaxation, which regulates the repression
455 or activation state of the regulator (13). Conformational changes may also permit transient
456 contacts of the regulator with DNA sequences flanking the T-N_{11}-A motif, which might also be

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457 affected by occupation by other DNA-interacting factors. The availability of the regulator in the
458 cell, the possibility of creating monomers or multimers to exert a regulatory effect on target
459 promoters, as well as the dynamic order in which different binding events take place, which
460 determines the appropriate regulatory response, could provide further levels of control. Our pull-
461 down results highlighted the existence of an intricate regulatory network engaging in possible
462 crosstalk, cooperation and/or interconnection between different transcriptional regulators
463 exerting an influence on *bsrA* expression and further on its targets. Thus, different factors control
464 LTTR interactions with DNA, providing specificity of recognition and correct timing of this
465 action.

466 Based on the presented results, we propose a model of the regulatory network engaging
467 BsrA in *P. aeruginosa* and its impact on bacterial physiology (Fig. 6). BsrA acts as the repressor
468 of genes involved in carbohydrate metabolism (*mqoA*, *acsA*) influencing the TCA cycle, the
469 availability of acetyl-CoA and overall cellular metabolism. In addition, BsrA regulates the
470 transcription of the uncharacterized sRNA *PA1112.1*, which is possibly involved in post-
471 transcriptional regulation of gene expression. Interestingly, besides autoregulation, the *bsrA* gene
472 is under the control of other LTTRs of *P. aeruginosa* (PA2551, PA3398 and PA5189) indicating
473 the ability to fine tune BsrA action in the cell. This multilevel regulatory network plays a role in
474 controlling carbohydrate metabolism (TCA cycle, acetate and pyruvate metabolism) and thus the
475 energetic status of the cell, which has implications for other functions such as cellular transport,
476 the response to antibiotic, phage infection, biofilm formation, virulence and overall survival
477 strategies. In line with this model, the induction of *bsrA* expression was observed in the presence
478 of antibiotics and also in *parA* and *parB* mutants characterized by growth retardation and defects
479 in chromosome distribution (103), which suggests the release of *bsrA* expression in response to

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480 stress and the need to redirect metabolism to cope with adverse conditions, that might be
481 manifested by a slowdown of bacterial growth.

482 MATERIALS AND METHODS

483 Bacterial strains, plasmids and growth experiments

484 Bacterial strains used and constructed in this study (listed in Table S1) were grown in LB or on
485 LB-agar at 37°C, and in M9 minimal medium supplemented with sodium citrate (0.25%) or
486 sodium acetate (20 mM) as the carbon source, with leucine (10 mM) added in the case of *P.*
487 *aeruginosa* PAO1161 *leu*$^{-}$ strains. For the selection of plasmids in *E. coli*, media were
488 supplemented with 10 µg/ml chloramphenicol, 50 µg/ml kanamycin or benzyl penicillin at a
489 final concentration of 150 µg/ml in liquid medium or 300 µg/ml in agar plates. For *P. aeruginosa*
490 strains, carbenicillin (300 µg/ml), rifampicin (300 µg/ml), kanamycin (250 µg/ml in liquid
491 medium; 500 µg/ml in plates) and chloramphenicol (75 µg/ml in liquid medium; 150 µg/ml in
492 plates) were applied as required.

493 For growth experiments, liquid media were inoculated with strains propagated on plates. These
494 cultures were grown overnight with shaking at 37°C, diluted 1:100 in fresh medium and then
495 incubation was continued. Bacterial growth was monitored by the measurement of optical
496 density at 600 nm (OD_{600}) at 1 hour interval. Competent *E. coli* cells were prepared by treatment
497 with $CaCl_2$ and transformation was performed according to a standard procedure (104).
498 Competent *P. aeruginosa* cells were prepared as described previously (105).

499 All plasmids used and constructed in this study are described in Table S1.

500 A *P. aeruginosa* PAO1161 Δ*bsrA* mutant was obtained by allele exchange (106). Competent
501 cells of *E. coli* S17-1 were transformed with plasmid pMEB14 (a derivative of suicide vector
502 pAKE600) to create the donor strain, and WT *P. aeruginosa* PAO1161 Rif^{R} was used as the

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503 recipient. The allele exchange procedure was performed as described previously (106, 107).
504 Verification of the obtained mutant strain was performed by PCR using primer pair #4/#7 (Table
505 S2).

506 Motility assays

507 Motility assays were performed as described previously (79), supplementing the swimming,
508 swarming, and twitching media, if necessary, with chloramphenicol (150 μg/ml) and IPTG (0.05
509 mM). To standardize the assays, all plates contained the same volume of the medium.

510 RNA isolation, RNA-seq and RT-qPCR

511 Total RNA was isolated from three independent replicate samples of *P. aeruginosa* PAO1161
512 overexpressing the *bsrA* gene as well as the control strain carrying the empty vector or *P.*
513 *aeruginosa* PAO1161 WT and the Δ*bsrA* strain. RNA isolation and analysis were performed as
514 described in Text S1.

515 Chromatin immunoprecipitation with sequencing

516 ChIP was performed according to the procedure of Kawalek et al. (108) with some
517 modifications, as described in Text S1.

518 Protein purification

519 *E. coli* BL21(DE3) transformed with pMEB10 encoding a His_6-BsrA fusion protein was grown
520 to exponential phase in autoinduction LB medium (Foremedium) containing 1% (v/v) glycerol
521 and 0.5% (w/v) NaCl. The cells were harvested by centrifugation, resuspended in phosphate
522 buffer (50 mM sodium phosphate, pH 8.0) supplemented with lysozyme (1 mg/ml), PMSF (1
523 mM) and benzonase nuclease (250 U, Sigma), then sonicated. His_6-BsrA was purified from the
524 cell lysate by chromatography on Ni-agarose columns (Protino Ni-TED 1000, Macherey-Nagel)
525 with 300 mM imidazole in phosphate buffer used for elution. The purification procedure was

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526 monitored by SDS-PAGE using a Pharmacia PHAST gel system. Fractions containing the
527 purified protein were dialyzed overnight in Tris buffer containing 5% (v/v) glycerol and stored in
528 small aliquots at −80°C.

529 *In vitro* protein-DNA interactions

530 The electrophoretic mobility shift assay (EMSA) was performed to determine the ability of
531 purified BsrA to bind to selected promoter regions of *P. aeruginosa* genes *in vitro*, as described
532 in Text S1.

533 Regulatory experiments with promoter-*xylE* fusions in *E. coli*

534 *E. coli* DH5α double transformants carrying pPT01 derivatives with the promoter regions of
535 selected *P. aeruginosa* genes fused to the *xylE* reporter gene plus pAMB9.37 (*lacI^Q-tac*p)
536 derivatives expressing the tested proteins were assayed for catechol 2,3-oxygenase activity (the
537 product of *xylE*) as described in Text S1.

538 Tests of kanamycin sensitivity

539 The effect of kanamycin on PAO1161 cells was tested using the carbon source screening
540 procedure (74, 89) described in Text S1.

541 DNA pull-down assay

542 Pull-down analysis was performed as described previously (108) with modifications summarized
543 in Text S1.

544 Data availability:

545 The raw RNA-seq and ChIP-seq data supporting the results of this article were deposited in the
546 NCBI's Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under
547 GEO Series accession numbers GSE163234 and GSE163233 (for release after manuscript
548 acceptance).

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549

550 **SUPPLEMENTAL MATERIALS**

551 **Text S1.** Materials and methods.

552 **Figure S1** Selected diagrams and charts presenting the phenotypic analysis of *P. aeruginosa*
553 PAO1161 WT, Δ*bsrA* mutant, and the strain overproducing BsrA.

554 **A.** Growth curves of the *P. aeruginosa* PAO1161 *bsrA* mutant and WT strains in LB and
555 M9+leucine+citrate at 37°C (leu^- strains).

556 **B.** Growth curves of the *P. aeruginosa* PAO1161 *bsrA* mutant and WT strains in M9+citrate
557 and M9+acetate at 37°C (leu^+ strains).

558 **C.** Selected pictures of swimming and swarming assays.

559 **D.** Growth curves of *P. aeruginosa* PAO1161 strains carrying pMEB63 ($lacI^Q$-*tac*p-*bsrA*;
560 BsrA overproducer) in L-broth with gradient (0-0.5 mM) of IPTG inducer.

561 **Figure S2** Comparison of the impact of *bsrA* and *bsrA-flag* overexpression on the growth of
562 cells in culture.

563 **A.** Growth curves of *P. aeruginosa* PAO1161 strain carrying pMEB99 expressing *bsrA-flag*
564 fusion grown in L-broth with gradient (0-0.5 mM) of IPTG inducer.

565 **B.** Growth curves of *P. aeruginosa* PAO1161 *ΔbsrA* strains carrying pABB28.1 (*tacp-flag*;
566 F-EV) and pMEB99 ($lacI^Q$-*tac*p-*bsrA-flag*; BsrA-F) in L-broth with 0.5 mM IPTG
567 (BsrA-F+; F-EV+) or without induction (BsrA-F; F-EV).

568 **Data set S1** Full RNA-seq data for BsrA+ and EV transcriptomes**.** Genes identified only in
569 PAO1161 strain but not in PAO1 are described as "not annotated (NA)".

570 **Data set S2** Results of RNA-seq analysis. List of 157 genes with altered expression identified by
571 comparison of the transcriptomes of the BsrA+ and EV strains [fold change (FC) ≤ -2 or ≥ 2,

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572 FDR ≤ 0.05]. The PseudoCap categories in the bold text were the most informative and were
573 used as the gene information presented in Fig. 2B. Genes identified only in strain PAO1161 but
574 not in PAO1 are described as "not annotated (NA)".

575 **Data set S3** Results of ChIP-seq analysis. 765 BsrA-FLAG ChIP-seq peaks with a fold
576 enrichment (FE) cut-off value of ≥ 2 obtained by the comparison of BsrA-FLAG ChIP samples
577 with negative control samples. Genes identified only in PAO1161 strain but not in PAO1 are
578 described as "not annotated (NA)".

579 **Data set S4** Proteins interacting with *bsrA*p in a pull-down assay, identified by mass
580 spectrometry analysis. Only proteins binding *bsrA*p but not to the control fragment are shown.

581 **Table S1** Bacterial strains and plasmids used and constructed in this study.

582 **Table S2** List of primers used in this study.

583

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590

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909

910 **Table 1 Genes of *P. aeruginosa* likely to be regulated by BsrA, identified by ChIP-seq**
911 **analysis.** Loci with BsrA binding site(s) in the promoter regions preceding the genes and
912 showing altered expression in response to a BsrA excess were considered to be directly
913 regulated.

Peak number	First gene of operon in PAO1161 (D3C65_)	First gene of operon in PAO1	Position of summit relative to start codon	Gene in PAO1161 (D3C65_)	Gene in PAO1	FC (fold change in RNA-seq)	FE (fold enrichment in ChIP-seq)	Gene description
188	07865	*PA3452*	-341	07865	*PA3452*	-3.86	6.05	malate:quinone oxidoreductase
79	03195	*PA0606*	-482	03195	*PA0606*	-2.87	2.73	AgtD, ABC

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								transporter permease
534	21160	*PA0952*	-244	21160	*PA0952*	-2.82	2.13	hypothetical protein
751	29570	*PA5445*	-89	29570	*PA5445*	-2.46	2.47	acetyl-CoA hydrolase/ transferase family protein
627	24675	*PA4542*	-54	24675	*PA4542*	-2.24	2.42	ClpB, chaperone protein
53	02095	*PA0396*	12	02095	*PA0396*	-2.17	2.82	PilT/PilU family type 4a pilus ATPase
508	20310	*PA1112.1*	-56	20310	*PA1112.1*	-2.12	12.70	non-coding RNA
11	00575	*PA0105*	-198	00575	*PA0105*	-2.03	3.36	CoxB, cytochrome c oxidase subunit II
405	16285	*PA1874*	-216	16285	*PA1874*	2.00	3.90	Ig-like domain repeat protein
188	07875	*PA3450*	-278	07875	*PA3450*	2.03	6.05	LsfA, 1-Cys peroxiredoxin
372	14370	*PA2231*	-139	14330	*PA2239*	2.04	4.77	PslI, glycosyltransferase family 1 protein
552	21955	*PA0805*	-28	21955	*PA0805*	2.08	2.13	hypothetical protein
180	07670	*PA3488*	-305	07670	*PA3488*	2.09	2.45	hypothetical protein
410	16485	*PA1838*	-7	16490	*PA1837*	2.10	2.02	DUF934 domain-containing protein
348	13300	*PA2440*	-374	13295	*PA2441*	2.10	2.45	hypothetical protein
389	15535	*PA2020*	-72	15535	*PA2020*	2.11	2.50	MexZ, transcriptional regulator
652	25380	*PA4673.1*	28	25380	*PA4673.1*	2.15	7.15	tRNA-Met
189	07895	*PA3446*	-31	07895	*PA3446*	2.16	5.54	NADPH-dependent FMN reductase
209	08850	*PA3266*	-21	08850	*PA3266*	2.16	2.70	CspA, cold-shock protein
481	18935	*PA1372*	-193	18940	*PA1371*	2.17	2.90	DUF2290 domain-containing protein
443	**17455**	***PA1656***	**-253**	17445	*PA1658*	2.20	4.09	TssC, type VI secretion system contractile sheath large subunit
				17450	*PA1657*	2.20		TssB, type VI secretion system contractile sheath small subunit
309	12080	*PA2667*	-148	12080	*PA2667*	2.22	2.95	MvaU, H-NS family transcriptional regulator
320	12420	*PA2602*	-74	12420	*PA2602*	2.31	2.07	3-mercaptopropionate dioxygenase
118	05080	*PA3981*	-46	5070	*PA3983*	2.32	2.02	HlyC/CorC family transporter
646	**25245**	***PA4648***	**-191**	25250	*PA4649*	2.00	6.09	CupE2, Pilin subunit

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				25245	*PA4648*	2.43		CupE1, Pilin subunit
276	11045	*PA2852.1*	64	11045	*PA2852.1*	2.51	2.26	tRNA-Ser
643	25115	*PA4624*	-16	25115	*PA4624*	2.53	2.12	cyclic diguanylate-regulated TPS partner B, CdrB
528	20980	*PA0985*	-471	20980	*PA0985*	2.82	2.58	pyocin S5
66	02635	*PA0499*	-89	02635	*PA0499*	2.93	8.75	probable pili assembly chaperone
389	**15540**	***PA2019***	**-94**	15540	*PA2019*	5.94	2.50	MexX/AmrA family multidrug efflux RND transporter periplasmic adaptor
				15545	*PA2018*	6.34		MexY/AmrB family multidrug efflux RND transporter permease subunit
				15550	-	8.27		transporter
379	14975	*PA2121*	-8	14975	*PA2121*	953.92	10.08	LysR family transcriptional regulator

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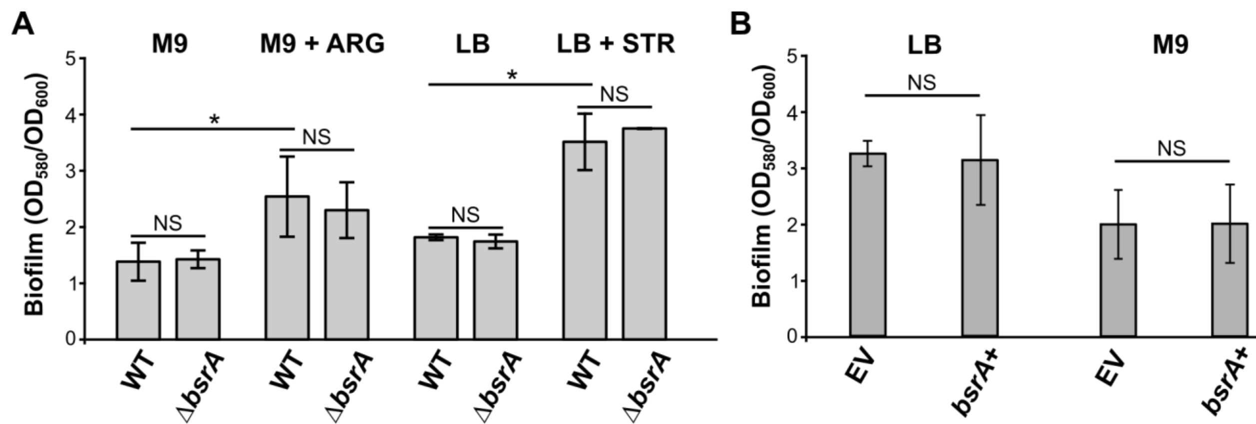

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919 **Figure 1 A lack or excess of BsrA does not affect biofilm formation by *Pseudomonas*
920 *aeruginosa* PAO1161.** Biofilm production in static cultures of (**A**) PAO1161 wild-type and the
921 Δ*bsrA* strain grown in M9 medium supplemented with citrate as the carbon source (with or
922 without arginine) or in LB medium (with or without 8 µg/ml streptomycin) for 48 h, and (**B**) the
923 strain carrying pMEB63 (*lacI*^{Q}-*tac*p-*bsrA)* overexpressing BsrA (*bsrA*+) and a control strain

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924 carrying empty vector (EV) pAMB9.37 (*lacI*^{Q}-*tac*p), grown in medium supplemented with 0.05
925 mM IPTG for 72 h. OD_{600} values were measured and biofilm formation was assessed by staining
926 with crystal violet, followed by measurement of OD_{580}. Data represent the mean OD_{580}/OD_{600}
927 ratios ± SD from 5 biological replicates. * p-value <0.05 in a two-sided Student's *t*-test. NS – not
928 significant (p-value > 0.05).

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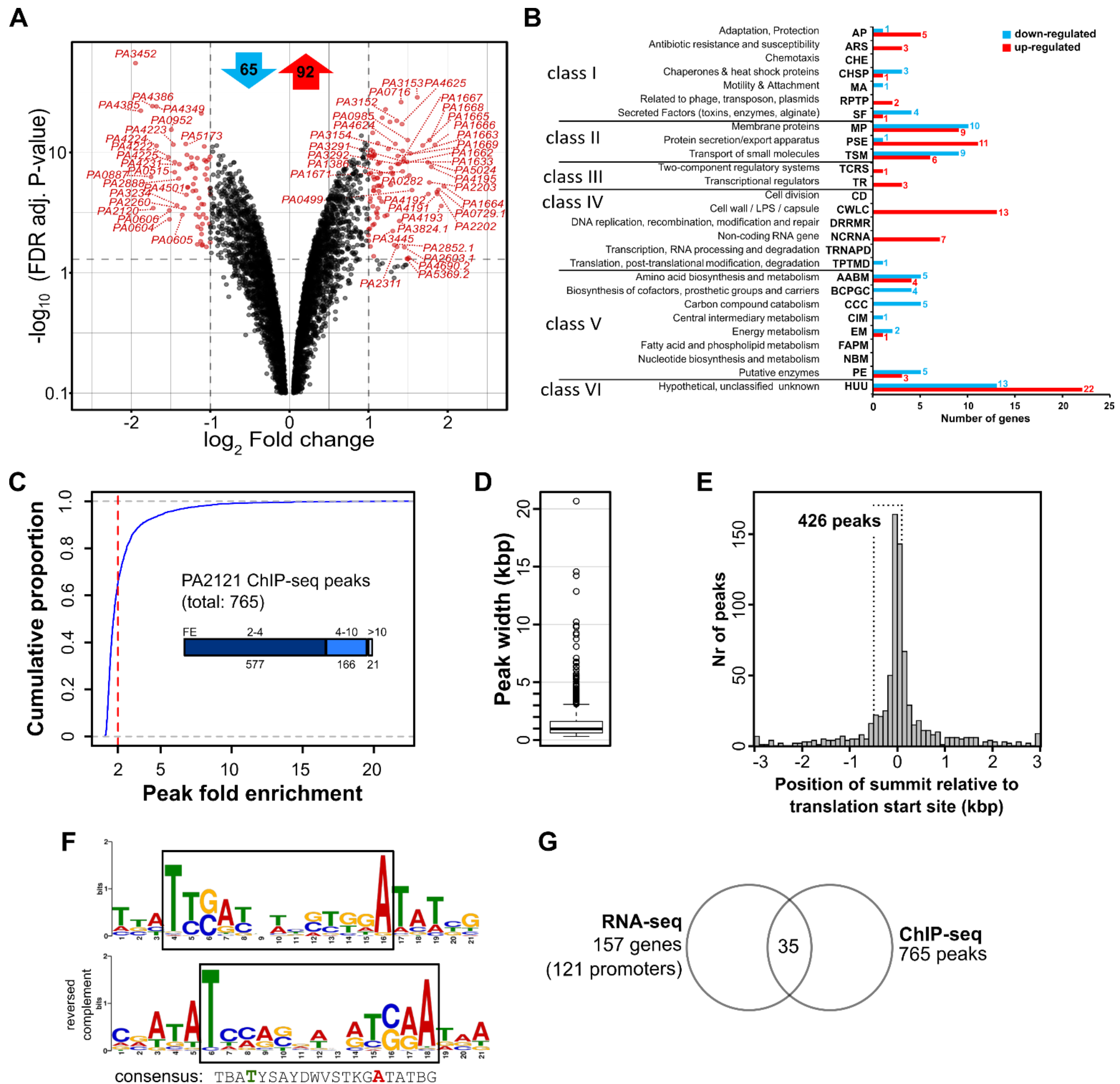

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932 **Figure 2 Identification of BsrA-dependent genes and binding sites for this transcriptional**

933 **regulator in *P. aeruginosa*.** Transcriptomes of PAO1161 cells carrying pMEB63 (*tac*p-*bsrA*,

934 overexpressing BsrA - BsrA+) or pAMB9.37 (*tac*p, empty vector control - EV), grown under

935 selection in LB supplemented with 0.05 mM IPTG were analyzed by RNA-sequencing. (**A**)

936 Volcano plot of RNA-seq data comparing the transcriptomes of BsrA+ and EV cells.

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937 Differentially expressed genes (Fold change >2 or < -2, and FDR-corrected *p*-value ≤ 0.01) are
938 indicated in red and the genes with the most significant changes in expression are named. For
939 clarity, genes with a *p*-value < 0.1 are not shown. The numbers of up- and down-regulated loci
940 are presented at the top in red and blue arrows, respectively. (**B**) Classification of loci with
941 altered expression in response to BsrA excess according to PseudoCAP categories (60). When a
942 gene was assigned to multiple categories, the most informative category was selected (in bold in
943 Data set S2). The PseudoCAP categories were additionally grouped into six classes (103, 109).
944 Red and blue bars correspond to the numbers of up- and down-regulated genes, respectively. (**C**)
945 Identification of BsrA binding sites in the *P. aeruginosa* genome. Cells expressing BsrA-FLAG
946 (or the control) were subjected to chromatin immunoprecipitation using anti-FLAG antibodies.
947 Reads obtained by sequencing of the ChIP DNA were mapped onto the PAO1161 genome (57)
948 and peaks were called using MACS2 with data from a mock-treated sample as the normalisation
949 control. The chart represents the distribution of fold enrichment (FE) values for the detected
950 peaks. A cut-off value of 2 is indicated by a red line. (**D**) Width distribution of BsrA ChIP-seq
951 peaks. (**E**) Distribution of the distance between ChIP-seq peak summits and the nearest start
952 codon. Bin width is 100 nt. Peaks with distances of > 3 kbp are grouped together in boundary
953 bins. (**F**) Sequence logo of the BsrA binding motif obtained by MEME (66). The reverse
954 complement of this logo and a proposed consensus sequence are presented below. B – C or G or
955 T, Y – C or T, S – G or C, D – A or G or T, W – A or T, K – G or T . The LTTR box (T-N_{11}-A)
956 is framed in black. (**G**) Overlap between RNA-seq and ChIP-seq results. A gene was classified
957 as likely to be directly regulated by BsrA if the ChIP-seq peak summit was located in the region -
958 500 to +100 from its start codon (or the start codon of the corresponding operon).
959

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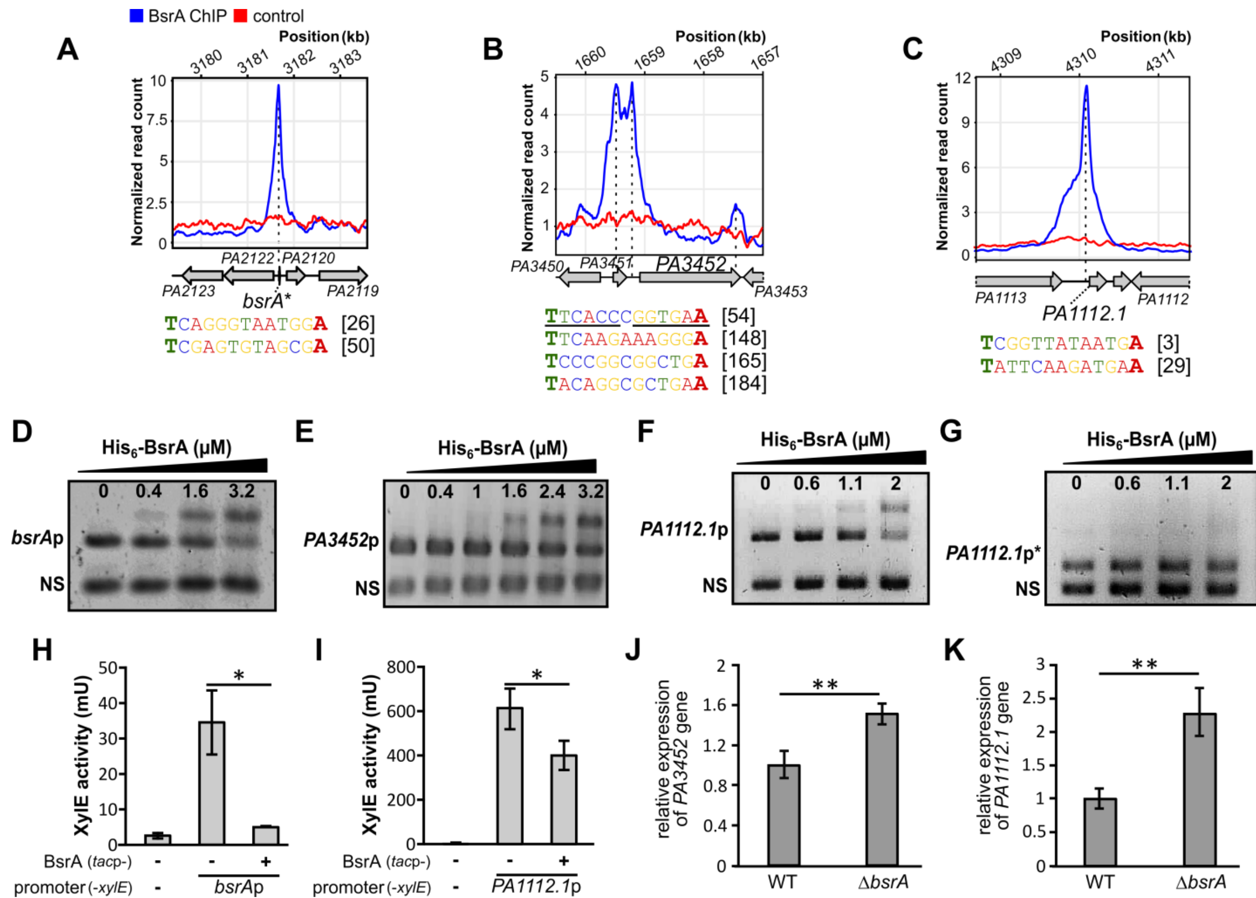

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961

962 **Figure 3 Direct regulation of target promoters by BsrA binding.** ChIP-seq signal over the

963 regions preceding the *bsrA* (**A**), *PA3452* (**B**) and *PA1112.1* (**C**) genes. The plots show

964 normalized read counts, averaged for ChIP replicates, for the indicated positions in the PAO1161

965 (CP032126.1) genome. Genes are represented as arrows and the names of the PAO1 orthologues

966 are shown for clarity. Sequences within the analyzed promoter fragments that correspond to the

967 T-N_{11}-A motif are presented below the plots, including their position relative to the start codon

968 (underlined sequences indicate a pseudo-palindrome). EMSA analysis of His_6-BsrA binding to

969 regions preceding *bsrA* (**D**), *PA3452* (**E**) *PA1112.1* (**F**) and truncated *PA1112.1*p (lacking 71 bp

970 containing the T-N_{11}-A motif) (**G**). DNA fragments (0.1 µM) were incubated with the indicated

971 amounts of His_6-BsrA and complexes were separated by electrophoresis on 1.5% (**DEF**) or 2.5%

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972 (**G**) agarose gels stained with ethidium bromide. A 199-bp fragment of empty vector pCM132
973 (labeled as NS) was used as a control of binding specificity and a competitor DNA. XylE activity
974 in *E. coli* DH5α double transformants carrying pMEB190 (*bsrA*p-*xylE*) (**H**) or pMEB232
975 (*PA1112.1*p-*xylE*) (**I**) plus pMEB63 (*lacI*^{Q}-*tac*p-*bsrA*) for BsrA overproduction (+) or control
976 plasmid pAMB9.37 (-). Strains were grown in selective LB. Data for cells carrying the promoter-
977 less pPTOI (-*xylE*) and pAMB9.37 are shown as background controls. The data represent the
978 means ±SD from three biological replicates. * indicates $p < 0.05$ in a Student's two-tailed t-test.
979 Relative expression (RT-qPCR) of *PA3452* (**J**) and *PA1112.1* (**K**) in WT and Δ*bsrA* cells from
980 exponentially growing cultures (OD_{600} 0.2) normalized to the reference gene *rpsL*. ** indicates p
981 < 0.01 in a Student's two-sided t-test assuming equal variance.
982

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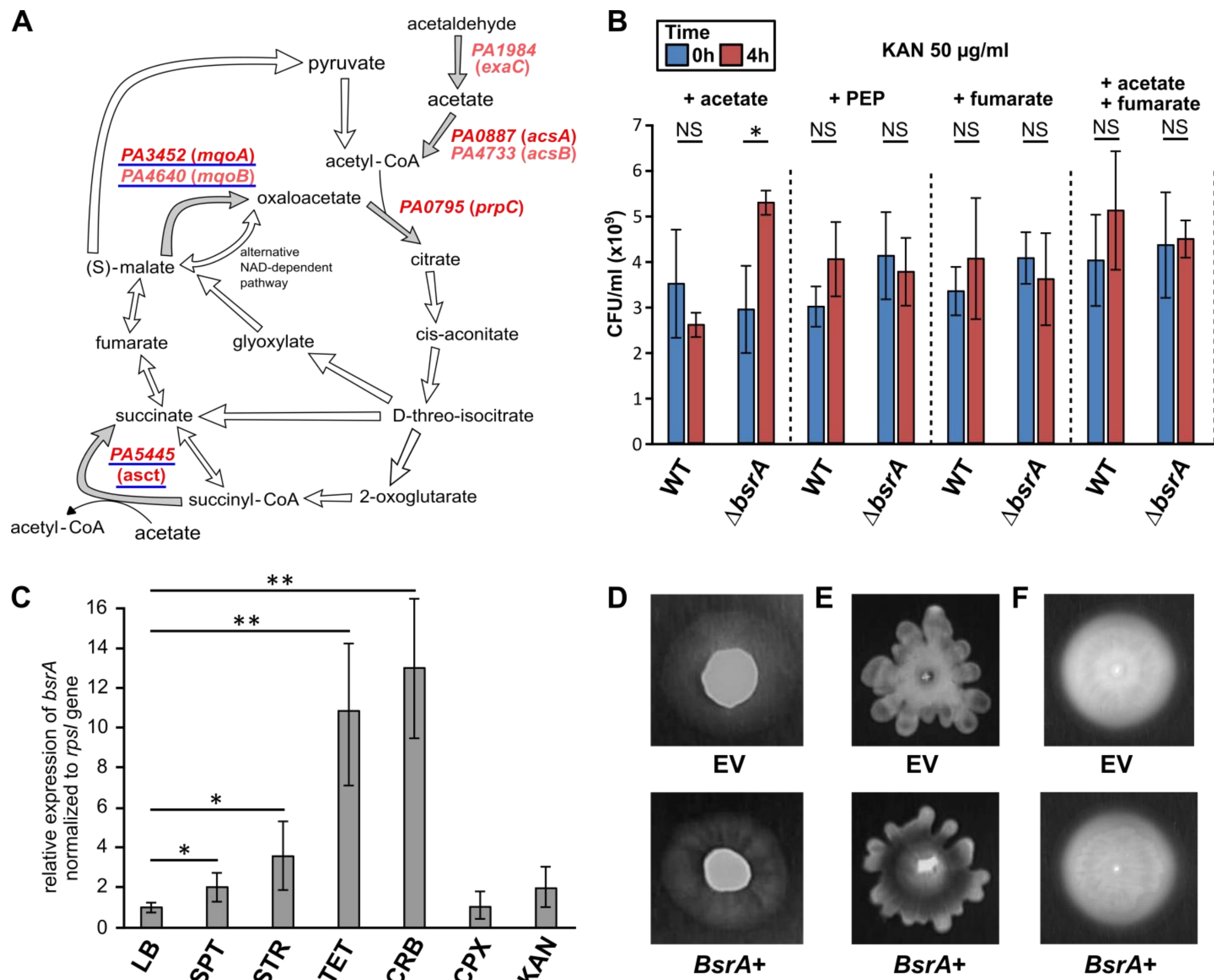

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984

985 **Figure 4 BsrA participates in the regulation of different processes in *P. aeruginosa***

986 **PAO1161.** (**A**) Scheme of the TCA cycle (71, 73, 82, 96). Genes identified as affected by BsrA

987 overproduction are indicated in red (dark red - FC > 2). Genes with BsrA binding sites in their

988 promoters are underlined. (**B**) Viable cell density (cfu/ml) of overnight cultures of PAO1161 and

989 the Δ*bsrA* mutant grown in M9 medium with kanamycin (50 µg/ml) and sodium acetate,

990 phosphoenolpyruvate (PEP) or fumarate added as the sole carbon source, in amounts adjusted to

991 maintain a total carbon concentration of 60 mM. * indicates a p-value of < 0.05 in Student's t-

992 test assuming equal variance. (**C**) Relative expression of *bsrA* in WT PAO1161 cells cultured in

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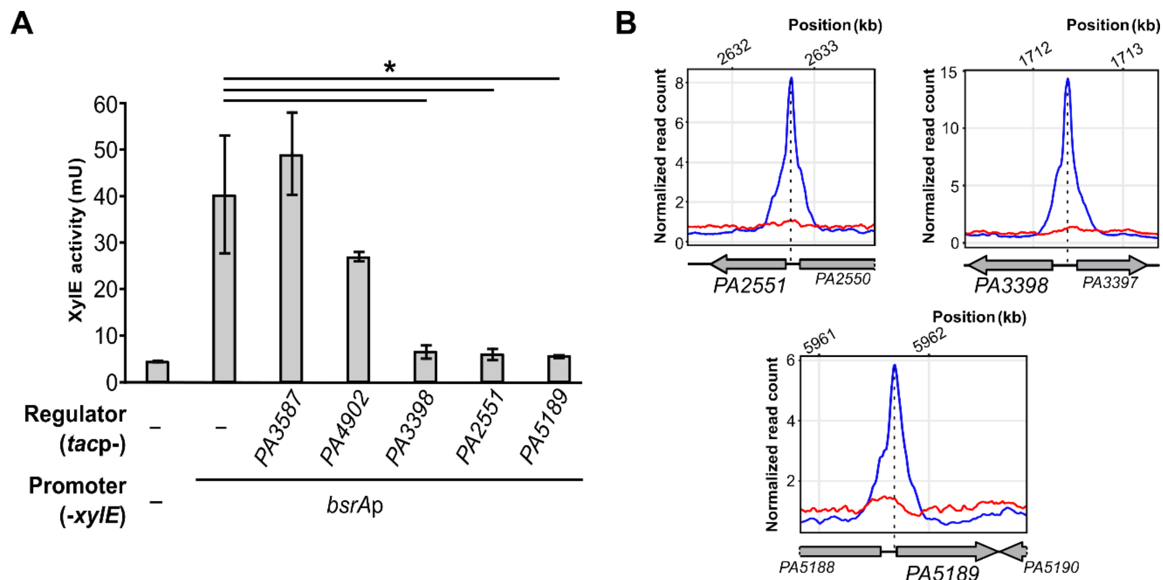
993 LB without antibiotic (LB) and with different classes of antibiotic added at sub-inhibitory
994 concentrations: spectinomycin 128 µg/mL (SPT), streptomycin 4 µg/mL (STR), tetracycline 4
995 µg/mL (TET), carbenicillin 32 µg/mL (CRB), ciprofloxacin 0.06 µg/mL (CPX) and kanamycin
996 10 µg/mL (KAN). * and ** respectively indicate *p*-values of < 0.05 and < 0.01 in a Student's
997 two-sided t-test assuming equal variance. Twitching (**D**), swarming (**E**) and swimming (**F**)
998 motility of strain PAO1161 carrying pMEB63 (overexpressing BsrA, *bsrA*+) or empty vector
999 pAMB9.37 (control, EV) treated with 0.05 mM IPTG.

1000



1001

1002 **Figure 5 Transcriptional regulators PA2551, PA3398 and PA5189 control expression of**
1003 ***bsrA*. (A)** XylE activity in double transformants of *E. coli* DH5α carrying promoter-less pPTOI
1004 or pMEB190 (*bsrA*p-*xylE*) plus vectors expressing the indicated genes under *tac* promoter
1005 control. Cells were grown in selective LB supplemented with 0.05 mM IPTG. Data represent the
1006 means ±SD from three biological replicates. * indicates $p < 0.05$ in a Student's paired two-tailed
1007 *t*-test. (**B**) ChIP-seq signals over regions preceding the *PA2551*, *PA3398* and *PA5189* genes
1008 encoding regulators repressing *bsrA* expression. The plots show normalized read counts,
1009 averaged for replicates, for the indicated positions in the PAO1161 (CP032126.1) genome.

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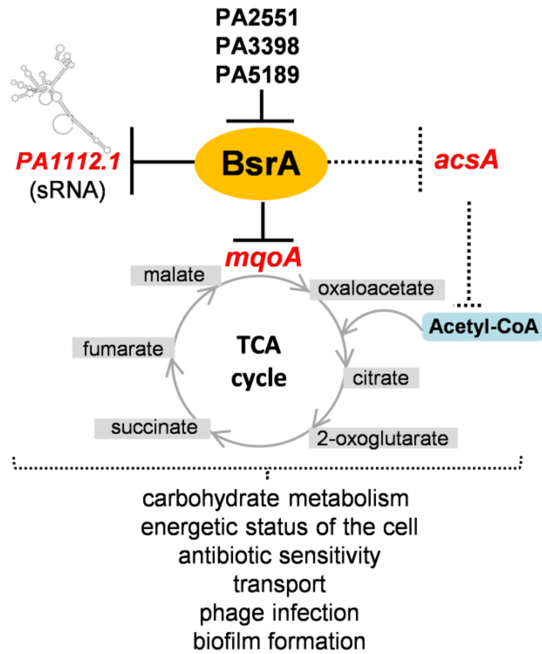

1010

1011 **Figure 6 The BsrA regulatory network in *P. aeruginosa* and its impact on bacterial**
1012 **physiology**. A black solid line indicates direct repression by this transcriptional regulator; a
1013 dotted line indicates direct and/or indirect involvement of BsrA in the control of gene expression
1014 and downstream processes.