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Pseudomonas aeruginosa adapts to octenidine via a combination of efflux and membrane remodelling

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20 Abstract.

21 Pseudomonas aeruginosa is an opportunistic pathogen capable of stably adapting to the antiseptic 22 octenidine by an unknown mechanism. Here we characterise this adaptation, both in the laboratory 23 and a simulated clinical setting, and identify a novel antiseptic resistance mechanism. In both 24 settings, 2 to 4-fold increase in octenidine tolerance was associated with stable mutations and a 25 specific 12 base pair deletion in a putative Tet-repressor family gene (smvR), associated with a constitutive increase in expression of the Major Facilitator Superfamily (MFS) efflux pump SmvA. 26 27 Adaptation to higher octenidine concentrations led to additional stable mutations, most frequently 28 in phosphatidylserine synthase pssA and occasionally in phosphatidylglycerophosphate synthase 29 pgsA genes, resulting in octenidine tolerance 16- to 256-fold higher than parental strains. Metabolic 30 changes were consistent with mitigation of oxidative stress and altered plasma membrane 31 composition and order. Mutations in SmvAR and phospholipid synthases enable higher level, 32 synergistic tolerance of octenidine.

34 Introduction

Antibiotic-resistant bacteria in healthcare mean increasing reliance on the use of antiseptics and disinfectants (biocides). Studies on high-consequence nosocomial infections caused by *Klebsiella pneumoniae*^{1,2}, *Enterococcus faecium*³, *Staphylococcus aureus*⁴ and *Pseudomonas aeruginosa*⁵, have shown increased tolerance to biocides, potentially making them problematic for infectionprevention and control (IPC).

40

P. aeruginosa, a Gram-negative opportunistic pathogen, causes outbreaks with high morbidity and
 mortality in neonatal units⁶, burns patients^{7,8} and persistently in cystic fibrosis sufferers⁹. Multi-drug
 resistant strains of *P. aeruginosa* are increasing^{10,11}, reducing treatment options. Low membrane
 permeability and multidrug efflux pumps cause higher biocide tolerance and resistance to many
 antibiotics¹².

46

47 IPC includes decolonising patients with antiseptic body washes, surface decontamination and hand 48 sanitisation for patients and clinical staff; all rely on biocides effectively killing bacteria. Cationic 49 biocides such as chlorhexidine, benzalkonium chloride and octenidine have relatively low toxicity and are effective against many Gram-positive and Gram-negative bacteria^{13,14}. Octenidine and 50 chlorhexidine, both gemini-surfactants with two cationic centres linked by an aliphatic hydrocarbon 51 chain, bind to the negatively charged cell membrane, disrupting it and leading to loss of the cell 52 wall¹⁵. Octenidine then interacts with the inner membrane causing chaotic lipid arrangement and 53 disruption of the cell envelope¹⁶. Increased biocide tolerance is linked to changes in membrane 54 charge/composition combined with efflux pump overexpression¹⁷. In K. pneumoniae, membrane 55 modification and an up-regulated Major Facilitator Superfamily (MFS) efflux pump, combine to 56 mediate resistance to chlorhexidine and cross-resistance to the last-resort antibiotic colistin². Little is 57 known about resistance to octenidine; previous studies have not shown increased tolerance.^{15,18}. 58 59 60 Previously we showed that P. aeruginosa can become stably tolerant to in-use-concentrations of octenidine in laboratory and simulated clinical settings⁵. The adaptation mechanisms were studied 61 62 here on a genetic and metabolic level, to understand the possible impact of octenidine-adaptation in

63 *P. aeruginosa* in the clinic.

64

66 **Results**

67 Stable mutations in an efflux regulator and phospholipid pathways following octenidine-68 adaptation

69 Stable mutations were identified in octenidine-adapted P. aeruginosa strains from various clades 70 (Supplementary Figure 1)compared to parental strains (Table 1), generated as outlined in 71 Supplementary Figure 2 and as described previously⁵. All adapted strains (8/8) contained mutations 72 in PA1283, a transcriptional regulator; 6 out of 8 contained SNPs in pssA (PA4693), a 73 phosphatidylserine synthase, or pgsA (PA2584), a CDP-diacylglycerol--glycerol-3-phosphate 3-74 phosphatidyltransferase. Additionally, two strains (CAS2 and CAS4) had SNPs in the signal sensor kinase PmrB (PA4777), a two-component regulator involved in biocide and colistin resistance^{2,19}. 75 Individual strains (CAS2 and PAO1) had mutations in hasR (PA3408), a heme-uptake outer 76 77 membrane receptor; and PA3328, a FAD-dependent monooxygenase, respectively.

78 PA1283 is divergently transcribed from PA1282, an MFS transporter, termed smvA in Salmonella enterica sv. Typhimurium and other organisms^{2,20}. PA1283, termed *smvR* for "*smvA*-regulator", is the 79 TetR-family repressor (TFR) regulating PA1282^{2,19}. Mutations in *smvR* included in-frame deletions in 80 4 strains (CAS2, CAS4, GH12 and PAO1), premature stop codons in 2 strains (NCTC 13437 and 372261 81 82 small-colony variant (SCV)) and a SNP in the remaining two strains (372261 large-colony variant 83 (LCV) and CAS3). A duplication in smvR (base pairs 306-317 and 318-329), coding for amino acids 84 102-106 (Ala-Ala-Ala-Leu-Met) and 106-110 (Met-Ala-Ala-Leu-Ile), was reduced to a singlet in GH12 and PAO1 deleting amino acids 106-109 (Met-Ala-Ala-Leu). Repeat experiments using the same 85 86 panel of 7 strains, showed this Δ 106-109 deletion in *smvR* in 11 of 14 adaptation studies and the 87 deletion of a single alanine (within the triple alanine motif of amino acids 102-104) occurred in 2 of 88 14 strains (Tables S2-S4).

89 PssA and PgsA are the first enzymes in divergent phospholipid biosynthetic pathways, starting with the common intermediate CDP-diacylglycerol²¹. Membrane remodelling is a common resistance 90 mechanism, usually linked to lipopolysaccharide (LPS) rather than phospholipids^{2,22}. The SNPs in *pssA* 91 encoded V222G (372261 LCV, CAS3 and GH12) and D240E/G (NCTC 13437 and CAS2 respectively). 92 93 SNPs in *pssA* were found repeatedly in octenidine-adaptation experiments with 100% of the 94 population having the alteration V222G in three strains and four further SNPs in this region, 95 including D240V, in a single strain (Supplementary Tables 1-3). Different mutations were found in the same strain background in repeat studies; e.g. PssA-V222G and SmvR- Δ 106-109 were found 96 97 separately in PAO1.

98 Efflux pump de-repression precede changes in phospholipid genes.

99 Multiple mutations are commonly seen during antimicrobial adaptation; either sequential changes 100 allowing survival at higher concentrations, or secondary mutations reducing impact on fitness (compensatory)²³. The presence of multiple mutations in *smvR*, *pssA* and *pgsA* were investigated 101 during adaptation, using population-mode BreSeq²⁴ on PCR products, to determine the frequency of 102 each mutation (Figure 1 and Supplementary Table 4). smvR mutations always appeared first, within 103 104 2 to 4 days (0.25x to 0.5x octenidine MIC), corresponding to a two-fold increase in the octenidine 105 population MIC⁵. In-frame deletions within *smvR*, notably deletion of amino acids 106-109, were 106 present in all strains, with the highest percentage (53.8-100%) occurring at passage 2 to 4 (0.75x to 107 1x MIC). Strains grown at octenidine concentrations at or above the parental octenidine MIC,

acquired SNPs in *pssA* and *pgsA* in 6 of 7 strains (except 372261). The conserved order of the
 mutations is noteworthy, giving synergistically increased MICs. In some populations, the *smvR* mutations are under-represented in the BreSeq analysis of final populations, notably passage 6 with
 NCTC 13437 where no *smvR* deletions are observed. This may suggest that these mutations exert a
 fitness cost when grown in mixed culture environments.

113 The same mutations occur in *P. aeruginosa* in a simulated clinical setting

114 P. aeruginosa strains were isolated from a clinically-relevant model of mixed-species biofilms in hospital sink traps⁵, exposed four-times daily to octenidine hand-wash (64 days), before the 115 116 octenidine was removed (27 days) and then reintroduced⁵. Mutations in *smvR*, *pssA* and *pqsA* from 117 P. aeruginosa isolates from six time points (day 0, 33, 61, 75, 96 and 110) were assessed by 118 population-mode BreSeq analysis (Supplementary Table 5). smvR showed multiple mutations in the 119 exposed population, including deletions of amino acids 106 – 109 and amino acid 102 alone; these 120 mutations again coincided with an increase in the octenidine MIC. When octenidine dosing was 121 paused (day 96), these mutations were not detected, likely because a different sequence type 122 became dominant, but reappeared on reintroduction (day 110). When present, the in-frame 123 deletions appeared at low frequencies within the population (4 – 8%), except at day 96, where $\Delta 102$ 124 was present in 96% of the population. No mutations were observed in *pssA* or *pqsA*; consistent with 125 the previous observation that mutations in these genes only occurred at octenidine concentrations 126 at/above the MIC (maximum octenidine concentration was sub-MIC (\sim 3µg/mL))⁵.

127 Constructing isogenic *smvR*, *pssA* and *pgsA* mutants in *P. aeruginosa*

128 Isogenic mutations were introduced into PAO1 using a novel recombineering method adapted from P. putida²⁵ (Supplementary Table 6). Mutations from octenidine-adapted strains (PssA V222G, PssA 129 130 D240G and D240E, PgsA T58M and SmvR A106-109) were introduced into PAO1, and SmvR-A106-131 109 double mutants generated with each PssA/PgsA SNP. Single mutants showed 2-4 fold increase in 132 octenidine tolerance, each SmvR-PssA double mutant showed a 32-fold increase and the SmvR-PgsA 133 double mutant a 16-fold increase, indicating a synergistic effect between mutations in the efflux 134 pump repressor and changes in phospholipid synthesis (Table 2). The increases in MIC for the single 135 SmvR-∆106-109 mutation was similar to that observed in PAO1, suggesting that the other mutations 136 observed in this background (PA3328 P51H) did not affect susceptibility to octenidine. The MIC 137 increase in the double mutants was lower than those observed for some of the adapted strains, 138 notably NCTC 13437 (256-fold increase). This is likely due to the influence of the strain background, as has been observed in our previous studies². Isogenic strains showed no increase in tolerance to 139 140 chlorhexidine or other biocides/antibiotics except for single mutants in PssA D240E and SmvR Δ106-141 109 which had 2-4-fold increase in MIC for chlorhexidine and alexidine. This differed from the octenidine-adapted strains, where most strains showed elevated resistance to chlorhexidine (6/7 142 143 strains), DDAB (2/7) and alexidine $(2/7)^5$, likely due to the additional mutations in the adapted 144 strains and/or the influence of individual strain backgrounds. PssA and PgsA variants showed no 145 attributable defect in growth rate or doubling time, compared with wild type PAO1 and SmvR Δ106-146 109 alone (although PssA D240E grew faster) (Supplementary Figure 3).

147 All of the single and double mutants were also tested for changes in MIC for existing antibiotics, 148 known to be substrates for one or more RND-family efflux pumps in *P. aeruginosa*. None of the 149 strains showed any significant difference in MIC, compared to the parental strain, with doxycycline, 150 nalidixic acid, meropenem, piperacillin, ceftazidime, ciprofloxacin or chloramphenicol151 (Supplementary Table 7).

152 Octenidine-adaptation constitutively increases *smvA* expression

153 Gene expression of smvA, smvR, pssA and pgsA was investigated in the isogenic mutants with and 154 without 0.25x MIC octenidine (Figure 2, Supplementary Tables 8-9). SmvA and SmvR-encoding genes 155 were constitutively upregulated in all strains containing SmvR Δ 106-109, in the presence or absence 156 of octenidine, confirming that this mutation de-represses the operon. The expression of pgsA was 157 significantly induced by 0.25x MIC of octenidine in all double mutants (1.4 – 7-fold), whilst pssA 158 expression was induced by octenidine in PAO1 PssA-V222G (1.3-fold) and in most double mutants 159 (2-8-fold), but not in PAO1 PssA-D240G/SmvR-Δ106-109. Levels of pssA were repressed in the PAO1 160 PssA-D240G single mutant (2-fold) compared to the wild type. Relatively small changes in expression 161 of pssA/pgsA are consistent with their tight regulation as branch points in the phospholipid 162 biosynthetic pathway²⁶, but the mechanism of gene regulation is not known. No significant changes to gene expression of smvA, smvR, pssA or pgsA were recorded in the WT PAO1 when challenged 163 164 with 0.25x MIC octenidine, possibly due to the low challenge dose.

165 Quantitative PCR of the octenidine-adapted strains also showed that smvA and smvR were 166 constitutively overexpressed with and without 0.25x MIC octenidine, irrespective of the type of 167 mutation present in smvR (Supplementary Table 10). Significantly higher overexpression of smvA and smvR were observed in all pssA/pgsA and smvR double mutants, compared to the single smvR 168 169 mutant. Interestingly, the PAO1 PssA-D240E/SmvR-Δ106-109 double mutant showed much higher 170 levels of *smvA* and *smvR* expression under octenidine challenge, than the PAO1 PssA-D240G/SmvR-171 Δ 106-109 mutant, suggesting a strong allelic effect of individual SNPs at this position. Changes in 172 pssA and pgsA expression levels were significant, but overexpression was not observed in all strains, 173 probably due to differing strain backgrounds.

Single and double *smvR* and *pssA/pgsA* mutations produce distinct changes in central carbon metabolism.

A ¹H high-resolution magic-angle spinning (HR-MAS) NMR approach with multi- and uni-variate analysis, adapted for use with *P. aeruginosa*²⁷, was used to detect changes in consumption/excretion of metabolites from isogenic mutants, with and without 0.25x MIC octenidine. This identified: 1) an inducible change in metabolism, occurring in PAO1 WT and all isogenic mutants; 2) constitutive and inducible changes in metabolism associated with some or all of the single isogenic mutants and; 3) a distinct metabolic strategy in the double mutants, not seen in the WT or single isogenic mutants.

182 In TSB, P. aeruginosa PAO1 relies on arginine and asparagine catabolism and consumption of formate, a key electron donor²⁸, during anaerobic respiration. Fermentation pathways are available, 183 184 but P. aeruginosa abstains from utilising glucose, abundant in TSB. For both the WT and all isogenic 185 mutants, octenidine-exposure induced a modest increase in formate, glutamate and alanine 186 consumption, triggered glucose, pyruvate, isoleucine and lysine consumption and cytosine, NADH 187 and tryptophan excretion (Figure 3; Supplementary Figures 4-5). Limited activation of fermentative 188 pathways was supported by increased production of acetate in WT PAO1 or lactate for certain 189 isogenic mutants.

Single isogenic mutations had little effect on most media metabolites (Figure 3; Supplementary
 Figures 4-5), except the excretion of lactate and consumption of succinate, in all octenidine-exposed

isogenic mutants but not WT PAO1. Cellular metabolite analysis reveals that all single mutants of SmvR, PssA or PgsA, showed significant ($p \le 0.05$) constitutive increases of acetate and decreases of glutamate levels compared with WT suggesting that acetate is retained in the cell and not excreted. These changes correlated with increases in cellular ethanolamine, ornithine and putrescine; Supplementary Figures 6-7). Ornithine can be decarboxylated into putrescine and this, together with the downstream product spermidine, can protect the outer membrane from oxidative and antibiotic stress by binding to LPS²⁹.

199 Cellular acetate, glutamate and ethanolamine levels returned to WT levels in all PAO1 SmvR 200 PssA/PgsA double mutants, with or without octenidine (Figure 3; Supplementary Figures 4-5). This 201 implies that the mutations work in synergy, directly or indirectly, to reverse the oxidative-stress 202 effect of each single mutation, and restore the catabolic strategy to WT, or to produce an 203 alternative, constitutive metabolic strategy. The octenidine inducible consumption and excretion of 204 metabolites to and from the media lends support for the latter. While consumption of succinate and 205 excretion of lactate was similar for double and single isogenic mutants, a distinct metabolic 206 phenotype could be described for the double isogenic mutants on exposure to octenidine, with 207 reduced consumption of alanine and consumption, rather than production, of acetate. There were 208 substantial increases in fumarate, tyrosine, methionine and valine excretion, while proline 209 consumption stopped, consistent with increased succinate dehydrogenase activity and use of the 210 glyoxylate shunt to bypass reactive oxygen species producing steps³⁰.

Therefore, both the constitutive and octenidine-inducible metabolic phenotypes of the single and double isogenic *P. aeruginosa* PAO1 mutants are distinct. Oxidative stress is both a constitutive feature of metabolism in the single isogenic mutants as well as being associated with octenidine challenge. In each case, different means are used to overcome oxidative stress.

215 Double *smvR* and *pssA/pgsA* mutations induce changes in signatures associated with 216 membrane remodelling.

217 The change in ethanolamine metabolism may be linked to phospholipid biosynthesis. The mutations 218 in the phospholipid pathway enzymes PssA and PgsA, altered cellular lipid metabolism in the 219 isogenic mutants compared to WT PAO1 (Supplementary Figures 7-10). Changes in acetate and 220 ethanolamine were negatively, and glutamate positively correlated with changes in lipids and N-221 acetyl-x moieties. In contrast to acetate, glutamate and ethanolamine, which only changed in the 222 single mutants, constitutive changes were detected in resonances assigned to lipid acyl chain 223 groups; most notably -CH₂- and -CH₃ and, to a lesser extent -CH=CH- as well as N-acetyl-x (1, 2 and 224 3). The identities of the three different N-acetyl groups could not be confirmed using the NMR 225 method. An increase in a second LPS feature (LPS (2)) was identified in the PAO1 SmvR∆106-226 109/PssA-D240E double mutant only (Supplementary Figures 7/9). LPS (1) and a resonance assigned 227 to lipid headgroup glycerol (1) suggested an octenidine-inducible effect in the double mutants and 228 WT. This indicates the lipid composition of the plasma and/or outer membrane is altered only when 229 mutations in SmvR are combined with and PssA V222G, PssA D240X or PgsA T58M and may, to some 230 extent, be induced by the presence of octenidine. The change in lipid composition is the most 231 notable change in metabolomic phenotype associated with the double isogenic mutants that display 232 synergistically elevated octenidine tolerance.

Cellular choline and betaine, both of which can act as osmoprotectants³¹, were changed in the isogenic mutants (Supplementary Figure 5), implying that osmotic stress may play a role in mutations selected by octenidine challenge. Betaine levels were generally lower in the isogenic mutants compared with WT, although PssA-D240X mutants were not affected to the same extent (Supplementary Figure 5). An increase in choline was observed only in the PgsA mutants (Supplementary Figures 5/10), implying that the role of choline in the phosphatidylcholine pathway, rather than as an osmoprotectant, had been affected by the PgsA-T58M mutation.

240 Overall, the octenidine-adapted strains showed broadly similar changes to the same cellular 241 metabolites (Supplementary Figures 11-13), but in several strains no constitutive (372261 SCV, CAS2, 242 CAS4, PAO1) or inducible changes (CAS4 and GH12) were found. Strains 372261 LCV, CAS3, GH12 243 and PAO1, showed very few changes whilst in NCTC 13437, 372261 SCV, CAS2, CAS3 there were 244 many changes. This implies that backgrounds and/or other adaptive changes can have a large impact 245 on metabolism. Interestingly, the PAO1 strain with the single SmvR mutation did not show a similar 246 pattern to the isogenic mutant, in terms of either signatures of metabolic stress (acetate, glutamate) 247 or the presence of some of the lipid metabolites (glycerol lipid head group (2) and N-Acetyl-x (2)), 248 despite the absence of mutations in PssA/PgsA. It is possible that the additional FAD-dependent 249 monooxygenase mutations in this strain have restored metabolic function.

250 Single mutations in SmvR and PssA/PgsA constitutively increase membrane order

251 Changes in the membrane, suggested by changes in cellular lipid and LPS components, were further investigated using two fluorescent dyes: Laurdan³² and diphenylhexatriene (DPH)³³, both of which 252 are probes reporting on membrane physical properties (Figure 4). The negligible correlation (R^2 = 253 254 0.00742) between DPH anisotropy and Laurdan Generalized Polarization (GP) indicate that the two 255 molecules report on different effects and, probably different components of the bacterial cell wall 256 (Supplementary Figure 14). Changes in DPH fluorescence anisotropy were modest although a 257 substantial increase was detected for the PAO1 PssA-D240E single mutant, both in the presence and 258 absence of octenidine (Figure 4B). No trends could be identified for single versus double isogenic 259 mutants, nor for individual metabolites and DPH fluorescence anisotropy, using a partial least-260 squares (PLS) regression model (Supplementary Figure 15).

261 GP was sensitive to both constitutive and induced changes in membrane order (Figure 4A). All single 262 SmvR and PssA/PgsA mutants showed a similar, modest increase in GP, indicating a more ordered membrane³² compared with WT PAO1. Double mutants, however, showed decreased GP compared 263 to PAO1 indicating a more disordered membrane. The addition of 0.25x MIC octenidine decreased 264 order in all strains except PssA D240E, in which it increased slightly. PLS regression of GP and relative 265 cellular metabolite levels in the isogenic strains ($R^2 = 0.429$, $Q^2 = 0.218$; Supplementary Figure 16) 266 267 identified seven metabolites, whose concentration correlated with the change in Laurdan GP (p < p268 0.05) (Figure 4C-H; Supplementary Figure 16). Metabolites likely associated with oxidative stress 269 (acetate, glutamate, ethanolamine and putrescine) correlated most strongly, followed by changes in lipids and an unidentified N-acetyl resonance (lipid CH₂, lipid CH₃, N-acetyl-x (1 and 4), glycerol (lipid 270 271 headgroup) (1), LPS (2 and 3)) (Figure 4; Supplementary Figure 16). Changes in lipid order are shown 272 to affect or be affected by both the metabolism of the cell and, more specifically, by lipid 273 metabolism. The relationship between these changes and increased octenidine tolerance is 274 discussed below.

Substantially decreased membrane order was observed for octenidine-adapted NCTC 13437, 372261
LCV and CAS2, whereas the parental strains did not show any induced effect on membrane order
(Supplementary Figure 17).

279

Transmission electron microscopy did not show gross differences in morphology between single and double mutants, although there was a trend towards a greater frequency of elongated (Supplementary Figure 18) and septating (Supplementary Figure 19) cells in the PAO1 PssA-D240G/SmvRΔ106-109 double mutants compared with the corresponding single mutants and wild type strain, but only in the presence of octenidine.

285

286 **Discussion**

287 Mounting evidence of decreased susceptibility to biocides commonly used in healthcare is 288 concerning given increased reliance on them to prevent infections with MDR organisms. In this study 289 we find that stable, increased tolerance to octenidine in *P. aeruginosa* is caused by increased efflux 290 and membrane modifications, with higher levels achieved where these two mechanisms of 291 resistance work in synergy (Figure 5).

292 Increased expression of efflux pumps is a common resistance mechanism for antibiotics and 293 biocides¹⁷. Stable mutations in regulators of multiple efflux pumps, commonly include TFRs, regulating MFS pumps (equivalent to SmvR) (e.g. QacR in Staphylococcus aureus)^{34,35}. We have 294 295 previously shown that the homologue of SmvR, the regulator of the MFS efflux pump SmvA, is mutated in response to chlorhexidine exposure in Klebsiella pneumoniae, Klebsiella oxytoca, 296 Citrobacter freundii and Salmonella enteritidis, leading to de-repression of SmvA^{2,19}. SmvA itself is 297 mutated in octenidine-adapted K. pneumoniae¹⁹. We found that the regulator SmvR was always 298 299 mutated and no mutations were observed in SmvA in octenidine-adapted *P. aeruginosa*; this likely 300 reflects different substrate binding between efflux pumps in different species. Deletion of a twelve 301 base-pair nucleotide tandem repeat (GGCGGCGCTGAT) encoding amino acids 106-109 (Met-Ala-Ala-302 Leu), outside of the DNA binding region in the canonical TFR, was found in all strains tested; this 303 resulted in at least 20-fold upregulation of SmvA and SmvR (Figure 5). Tandem repeats are 304 disproportionately frequent in genes whose products are involved in stress response, allowing for an immediate response by mechanisms including slip strand mispairing or polymerase slipping³⁶. 305 306 Indeed, we found that the removal of one of the 12 bp tandem repeats in SmvR always occurred as 307 an initial mutation, within 2 to 4 days following exposure to low levels of octenidine, in all strains 308 tested. As this was seen in both lab-adaptation studies and repeatedly in selected isolates from the 309 sink trap model^{37,5}, this is likely to be a common initial response of *P. aeruginosa* to low levels of 310 octenidine and/or other stresses (Figure 5). The multiple mutations found in SmvR, all of which 311 affect the regulation of SmvA, also suggest this is a hypermutable region, allowing the organism to 312 adapt quickly to stress.

In *K. pneumoniae*, docking studies showed that SmvA interacts with both chlorhexidine and octenidine as potential substrates¹⁹. *P. aeruginosa* SmvA shows 40% homology with *K. pneumoniae* SmvA which, taken together with its upregulation following octenidine-adaptation, suggests SmvA may transport cationic biocides in *P. aeruginosa*, also (Figure 5). However, SmvA was not significantly upregulated in WT PAO1 in the presence of 0.25x MIC octenidine, differing from *K.*

pneumoniae where clear upregulation was observed^{2,19}. Higher levels of octenidine may, however, 318 319 show an increase in smvA transcription. PAO1 SmvR transposon mutants showed an increase in 320 chlorhexidine MIC, but not octenidine (Supplementary Table 11), suggesting possible differences in 321 substrate specificity of the two pumps/regulators. Although there is clear evidence for SmvA's role in 322 mediating cationic biocide resistance in different Gram-negative bacteria, questions remain around 323 how this affects efflux of compounds out of the cell. In Salmonella, SmvA is proposed to work in 324 conjunction with one or more outer membrane proteins, such as TolC and/or OmpW, to mediate export of the substrate methyl viologen³⁸. We did not see significant changes in MIC or MBC for 325 326 octenidine or chlorhexidine, using transposon mutants in any of the characterised outer membrane 327 proteins associated with efflux pumps in PAO1 (OprJ, OprM, OprN, OpmD; Supplementary Table 11). 328 This suggests that if there is an outer membrane component involved in SmvA-mediated efflux, it is 329 not within this set of known OMP efflux components or that the function is redundant, with no 330 single OMP mutant having a noteworthy impact on MIC (Figure 5). Other efflux pumps have been previously implicated in mediating resistance to chlorhexidine, notably MexCD OprJ³⁹. However, a 331 PAO1 derivative overexpressing MexCD-OprJ due to an insertion in nfxB (strain K1536³⁹) did not 332 333 show an increased MIC for octenidine (Supplementary Table 11).

334 Betaine levels were decreased in most single and double mutants, with a significantly increased 335 expression of SmvA, in the presence and absence of octenidine. Choline-derived, glycine-betaine can 336 be accumulated in cells as an osmoprotectant³¹. SmvA was originally characterised as a pump for methyl viologen²⁰, a small di-cation, and subsequent studies demonstrated a role in mediating 337 increased resistance to other cationic antiseptics and biocides^{2,19}. It is possible that glycine-betaine, 338 339 itself a quaternary ammonium cation, may also be effluxed by SmvA, resulting in reduced levels of 340 betaine in SmvA-overexpressing strains (Figure 5). Choline levels were only modified in isogenic 341 mutants with mutations in PgsA, most likely as a consequence of altered phosphatidylcholine 342 metabolism. Further studies of SmvA substrate specificity will confirm whether SmvA effluxes a 343 range of di-cations including octenidine, chlorohexidine and/or glycine-betaine in *P. aeruginosa*.

344 Mutations in phospholipid biosynthetic genes also affect octenidine tolerance of *P. aeruginosa*. The function and regulation PssA and PgsA have been well described in *E.coli*^{21,26}. PssA 345 346 (phosphatidylserine synthase A) converts the common intermediate, CDP-diacylglycerol, to 347 phosphatidylethanolamine (PE), whereas PgsA (phosphatidylglycerophosphate synthase A) is the 348 first step in the parallel pathway generating phosphatidylglycerol (PG). P. aeruginosa PgsA shows 349 high protein homology (60%) with E. coli PgsA, whereas PssA shows low conservation outside of the 350 active PssA domain (overall 29% protein sequence homology) with six predicted transmembrane 351 domains present only in *P. aeruginosa* PssA. This suggest a different mechanism of regulation to that seen for *E.coli* PssA, which transiently associates and dissociates with acidic phospholipids in the 352 membrane⁴⁰. Residues consistently mutated in *P. aeruginosa* PssA, V222 and D240, are outside of 353 354 the CDP-alcohol phosphatidyltransferase domain and located either side of the fifth predicted 355 transmembrane domain. As PssA regulation appears different in P. aeruginosa, we cannot define 356 whether the mutations directly affect enzyme function by modulating interactions with the 357 membrane, or influence expression/transcript stability of this pivotal enzyme. The T58M SNP in PgsA 358 is within the predicted CDP-alcohol phosphatidyltransferase domain and may directly influence 359 enzyme activity.

360 Cell surface modification is a common mechanism of resistance to cationic compounds, linked especially to modified lipid A and a more neutral membrane that may decrease binding of the 361 cations^{22,41}. Changes to the phospholipid composition have not been previously described as a 362 363 mechanism of cationic biocide/antimicrobial peptide (AMP) resistance in Gram-negative bacteria. 364 Studies in S. aureus have shown phospholipid composition modulates AMP resistance by increase or decrease in membrane fluidity or order⁴². In *S. aureus*, resistance to daptomycin, a membrane-active 365 lipopeptide antibiotic analogous in function to cationic biocides, is caused by a loss of function 366 mutation in PgsA⁴³ or gain of function mutations in cardiolipin synthase⁴⁴, resulting in a net decrease 367 368 in anionic PG in the membrane, due to accumulation of glycolipids and phosphatidic acids upstream 369 of PgsA. The mutations in P. aeruginosa PgsA and PssA are also likely to affect the ratio of 370 phospholipids in the membrane, possibly also leading to a decrease of PG (Figure 5). What the exact 371 membrane changes are and whether these changes directly affect binding/release of octenidine 372 from the bacteria, consistent with recently described mechanistic descriptions of octenidine 373 activity¹⁶ or mediate effects indirectly by modulating function of one or more membrane proteins 374 (including SmvA) remains to be determined.

375 Surprisingly, mutations in PssA and PgsA have a similar metabolomic effect on the cell to those of 376 SmvR mutations. Individually, they give only 2 to 4-fold increase in octenidine tolerance but these 377 were synergistic when combined, leading to elevated resistance. All isogenic single mutants showed 378 a more ordered membrane compared to WT, although this may be achieved through different 379 mechanisms. Constitutive changes in carbon metabolism present in the single mutants were lost in 380 the double mutants, and membrane order decreased, especially in the presence of octenidine. 381 Although this is reminiscent of the WT, the double mutants respond very differently to octenidine 382 challenge compared with either the WT or single mutants, consistent with an enhanced glyoxylate 383 shunt. In octenidine-adapted strains, mutations in SmvR always occurred first and mutations in PssA 384 or PgsA were never observed in isolation; these mutations only occurred at octenidine 385 concentrations higher than the WT MIC, in the presence of overexpressed SmvA (Figure 5). Changes 386 in acetate and glutamate levels (consistent with dissipation of constitutive, oxidative stress) and a 387 decrease in membrane order to wild type levels, supports the hypothesis that these mutations are 388 compensatory, rather than simply being driven by increased selective pressure. Alone, increased 389 numbers of SmvA pumps may not have the proton gradient to efflux effectively, functioning as a 390 proton antiporter, whilst PssA and PgsA alteration of lipid environment may confer only limited 391 resistance to octenidine. Together, PssA/PgsA may create an improved membrane environment for 392 glyoxylate shunt activity, increasing proton gradients and enhancing the efflux activity of SmvA. The 393 inducible nature of the changes in metabolism associated with this pathway support the hypothesis 394 of enhanced efflux, as without octenidine presence, maintenance of the proton motive force would 395 not be required.

396 Apart from an elevated glyoxylate shunt in response to octenidine challenge, the change in lipid 397 composition is the most notable metabolomic phenotype associated with the double isogenic 398 mutants. This indicates that the lipid composition of the plasma membrane is only stably altered 399 when mutations in SmvR and one of PssA V222G, PssA D240X or PgsA T58M are combined. Changes 400 in lipid acyl resonances, with increases of -CH=CH-, -CH₂ and -CH₃ in the double mutants, correlated 401 with the decreases in membrane order compared to the single mutants. Changes in phospholipid 402 composition and membrane order associated with altered headgroup or saturation and length of acyl chains, are known to affect membrane protein folding and consequently protein function^{45,46} 403

404 including both efflux pumps and succinate dehydrogenase⁴⁷. MFS efflux pump LmrP in *E.coli* was 405 found to depend on the presence of PE to form structural intermediates required for the transport 406 process⁴⁸. The dimerization of EmrE, an *E. coli* SMR efflux pump, into active transporters is also 407 affected by the lipid composition of the membrane⁴⁵. Given the evidence of direct interactions 408 between membrane proteins and specific phospholipids and assuming the large increases in *smvA* 409 transcript translates into concomitant increases in protein content, it is likely that overexpression of 410 the efflux pump also influences membrane lipid structure and *vice versa* (Figure 5).

411 We have shown that depressed efflux pump expression in synergy with lipid modification, increase 412 the tolerance of *P. aeruginosa* clinical isolates to octenidine. This is the first time that this 413 mechanism of synergy has been demonstrated and the first time that stable adaptation to 414 octenidine has been described. We do not see loss of fitness of double mutants or adapted strains as 415 shown by growth curves and *Galleria mellonella* infection⁵. We found the same TFR mutations in our simulated clinical setting, but we did not find mutations in phospholipid synthesis pathways, under 416 417 the conditions used, likely due to lower (sub-MIC) selective pressure in the sink trap model⁵. In other 418 clinical settings, such as during decolonisation of the skin using octenidine at 0.3% (3 mg/mL), P. 419 aeruginosa might be exposed to higher octenidine concentrations, possibly leading to selection 420 pressure for mutations in both *smvR* and phospholipid biosynthetic genes. This could lead to the 421 type of synergistic adaptations we have described here and elevated levels of octenidine tolerance. 422 We did not find any indication of cross-resistance to antibiotics in our isogenic mutants, contrasting 423 with previous results showing that octenidine-adaptation can select mutants with increased resistance to clinically-relevant antibiotics through mutations in other membrane modification 424 systems, such as PmrB (as seen in octenidine adapted Cas2 and Cas4) ⁵. Acyl-chain remodelling e.g. 425 saturation and chain length as induced here, is also a known mechanism of resistance to 426 antimicrobial peptides AMPs⁴⁹ and we cannot rule out the possibility that cross resistance to such 427 host-defences might also result from this type of selection (Figure 5). 428

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447 Author contributions

448 LIB, AJM and JMS conceived of the study. LIB designed, performed, analysed and interpreted the 449 majority of the molecular and microbiological research. PMF performed and analysed the 450 biophysical experiments. PMF and MC performed and analysed the membrane order and fluidity 451 experiments. MC and VP performed and analysed gPCR on adapted strains. PMF, MC and VP 452 performed and analysed the NMR metabolomic experiments. PF and LA performed electron 453 microscopy experiments and data analysis with RAF. MEW performed the whole genome sequence 454 analysis, generated the phylogenetic tree and carried out additional MIC assays. MJS performed 455 additional adaptation studies and designed Supplementary Figure 2. PF designed Figure 5. AJM and 456 JMS interpreted the data and edited the manuscript. LJB, AJM and JMS wrote the manuscript. The 457 manuscript was reviewed by all authors.

458 **Competing Interests**

459 The authors declare no competing interests.

460 Methods

461 **Bacterial strains and Reagents**

462 The P. aeruginosa strains are shown in Supplementary Table 12 and a phylogenetic tree in Supplementary Figure 1. Transposon mutants came from the Manoil lab^{50,51} and from Keith Poole 463 (Queen's University, Canada). The adaptation process was as described in Shepherd *et al*⁵ and 464 465 Supplementary Figure 2 and was carried out twice. In brief, a selection of 7 reference (PAO1) and 466 MDR strains, representative of the two main groups of *P. aeruginosa* strains defined previously⁵² 467 were selected for the study. Strains were exposed to octenidine dihydrochloride in Tryptic Soy Broth 468 (TSB), starting at $2\mu g/mL$ (0.25 – 0.5 x MIC) and grown for 2 days prior to subculture in double the 469 concentration of octenidine, until the strain either failed to grow or the concentration reached 470 64µg/mL. Populations from these studies were sampled throughout the passaging and as a final 471 population and these were used for BreSeq analysis (see below). Strains were serially passaged 10 472 times on Tryptic Soy Agar (TSA) plates in the absence of octenidine. The minimum inhibitory 473 concentration of octenidine for single colonies was checked and isolates showing elevated resistance 474 to octenidine (for 372261 a small colony variant, SCV, and a large colony variant, LCV) were sent for 475 whole genome sequencing.

All strains were grown at 37°C on TSA or TSB (Oxoid, 1896417, UK), unless specified. Biocides and
antibiotics used in this study [octenidine dihydrochloride, chlorhexidine digluconate,
didecyldimethylammonium bromide (DDAB), alexidine dihydrochloride, amikacin, colistin and
tobramycin] were purchased from Sigma-Aldrich (Steinheim, Germany), except octenidine
dihydrochloride (supplied by Schülke & Mayr, Norderstedt, Germany).

Exposure of sink trap populations to octenidine, isolation and characterisation of *P. aeruginosa* strains was described previously⁵. In brief, a sink trap taken from a UK hospital was flushed for 30 s four times a day at a flow rate of 4 L/min with the addition of 2mL octenidine bodywash containing 0.3% octenidine. The octenidine dosing continued for 63 days, was paused for 27 days and then resumed for a further 20 days. 100 μ L of water from the sink trap were plated on cetrimide-nalidixic acid agar (Oxoid Ltd, Basingstoke, UK) and incubated at 37°C overnight to select for *P. aeruginosa*. Cell populations from days 0, 33, 61, 75, 96 and 110 were used for MIC and BreSeq analyses.

488 Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MICs) were measured using the broth microdilution method in polypropylene plates (Greiner) as described previously¹. One hundred microlitres of 1 x 10⁵ colony-forming units/mL in TSB were mixed with 100 μ L of the diluted biocide/antibiotic, and optical density at a wavelength of 600 nm (OD₆₀₀) was measured after 24 hours of static incubation at 37°C. MIC was defined as the lowest concentration of biocide/antibiotic at which no growth was observed (OD₆₀₀ < 0.1 after subtracting the blank).

495 Next Generation Sequencing and Sequence Analysis

496 DNA was purified using a Wizard genomic DNA purification kit (Promega, Wisconsin, US). DNA was 497 tagged and multiplexed with the Nextera XT DNA kit (Illumina, San Diego, US) and sequenced by 498 PHE-GSDU (Public Health England Genomic Services and Development Unit) on an Illumina (HiSeq 499 2500) with paired-end read lengths of 150 bp. A minimum 150 Mb of Q30 quality data were 500 obtained for each isolate. FastQ files were quality trimmed using Trimmomatic⁵³. SPAdes 3.1.1 was

used to produce draft chromosomal assemblies, and contigs of less than 1 kb were filtered out⁵⁴. 501 FastQ reads from chlorhexidine-exposed isolates were subsequently mapped to their respective WT 502 pre-exposure chromosomal sequence using BWA 0.7.5⁵⁵. Bam format files were generated using 503 Samtools⁵⁶, and VCF files were constructed using GATK2 Unified Genotyper (version 0.0.7)⁵⁷. They 504 505 were further filtered using the following filtering criteria to identify high-confidence SNPs: mapping quality >30; genotype quality >40; variant ratio >0.9; read depth >10. All the above-described 506 sequencing analyses were performed using PHE Galaxy⁵⁸. BAM files were visualized in Integrative 507 Genomics Viewer (IGV) version 2.3.55⁵⁹. Whole genome alignment and phylogenetic tree generation 508 509 were performed using progressive alignment in Mauve Version 20150226 build 10. Tree visualisation 510 was performed in FigTree Version 1.4.3.

511 **BreSeq**

512 Colony PCR using primers listed in Supplementary Table 6 (LBOL191 and 192 (pssA), LBOL193 and 513 194 (smvR), and LBOL315 and 316 (pgsA)) to amplify the complete genes was performed on total 514 cultures for each passage and strain. PCR products were purified (Qiagen PCR purification kit, Hilden, 515 Germany), eluted in 30 µL ddH₂O and quantified using Invitrogen[™] Qubit[™] Fluorometer dsDNA kit. 516 To increase read depth coverage, PCR products from all three reactions per time point and strain 517 were mixed to contain 20 µg/mL DNA from each reaction and sent for Illumina Sequencing at Public Health England NGS sequencing service. Resulting contigs with a coverage of >15k were compared to 518 the gene sequence of PAO1 using BreSeq polymorphism mode⁶⁰ with a cut-off P-value of 0.01 to 519 520 identify the percentage of the population that had a given SNP or deletion over time. This method 521 uses a maximum likelihood analysis to identify the prevalence of SNPs or small deletions within a 522 bacterial population. Each result was manually checked for false positives according to the BreSeq 523 protocol on the Barrick lab website 524 (http://barricklab.org/twiki/pub/Lab/ToolsBacterialGenomeResequencing/documentation accessed 525 between October 2017 and June 2019).

526 **Recombineering**

The construction of isogenic strains in *P. aeruginosa* was adapted from Aparicio *et al*²⁵ and Nyerges 527 et al^{61} . PAO1 was selected for the recombineering work due to its relative drug sensitivity and 528 availability of resistance markers for maintenance of plasmids. Oligos (Supplementary Table 6) were 529 designed using MODEST⁶² to include single SNPs or a short 12 bp deletion. Standard desalted 530 531 ultramers were from Integrated DNA Technologies (IDT, Leuven, Belgium) with phosphorotioate bonds on the first two and last two nucleotides. PAO1 cells were made electrocompetent by washing 532 3 times in 300 mM sucrose before being transformed with pSEVA658-ssr⁶³ (Ec2 setting: 2 mm-gap 533 534 cuvette, 2.5 kV, 25 μ F, 200 Ω , in a BioRad MicroPulser, Watford, UK) and selected on 30 μ g/mL 535 gentamicin/TSA. A fresh colony was inoculated into TSB/gentamicin. The overnight culture was diluted to OD₆₀₀ 0.2 in 20 mL TSB/gentamicin. At OD₆₀₀ 0.4-0.5 1 mM XyIS effector 3-methylbenzoate 536 537 (3MB) was added for 30 minutes at 250 rpm and 37°C to induce expression of Ssr. Cells were made 538 electrocompetent and 1 μ L 100 μ M oligo transformed as above. Colonies were selected on 512 539 μ g/mL octenidine TSA plates. Resulting colonies were screened by Sanger Sequencing (SNPs, 540 Genewiz, UK) or meltcurve analysis (12bp deletions) using primers listed in Supplementary Table 6. 541 Meltcurve analysis (60.0°C to 95.0°C recorded every 0.3°C) was performed on a StepOne Plus 542 (Applied Biosystems, UK). Positive colonies were checked for off-target mutations via NGS (see 543 above). Double mutants were constructed by recombineering LBOL365 into already constructed

pssA/pgsA SNP mutants still containing pSEVA658-ssr. Mutants were regularly checked for reversion
 by meltcurve and Sanger Sequencing.

546 **Growth curves**

547 Three single colonies grown in TSB at 37°C 250rpm overnight were diluted to OD₆₀₀ 0.01 in TSB and 548 observed at OD₆₀₀ every 30 minutes for 24 hours at 37°C static growth. Differences in doubling time 549 and intrinsic growth rate were calculated using growthcurver in R version 3.5.1, ANOVA was 550 performed in in GraphPad Prism version 6.04 for Windows, GraphPad Software, La Jolla California 551 USA.

552

553 RNA extraction and quantitative PCR

554 qPCR was used to measure the expression of pssA, pgsA, smvR and smvA in the octenidine-adapted 555 and isogenic PAO1 mutants using primers listed in Supplementary Table 6 as described previously¹. 556 Triplicate overnight cultures grown in TSB were back diluted to an OD₆₀₀ of 0.1, and exposed to 0.25 557 x MIC octenidine or no octenidine for 30 minutes. Cells were then harvested using RNA protect 558 bacteria reagent (Qiagen, Hilden, Germany) at mid-log phase (OD₆₀₀ 0.5), and RNA extracted using 559 the RNeasy minikit (Qiagen), including on-column DNase treatment according to the manufacturer's 560 instructions. In addition, 5 µg RNA was treated with a DNA-free kit (Ambion, UK), of which 0.2 µg 561 RNA was reverse transcribed using the SuperScript III first-strand synthesis system for RT-PCR 562 (Invitrogen, UK) according to the manufacturer's instructions. Samples were checked for DNA 563 contamination by performing qPCR on negative reverse transcription reaction for each sample. qPCR 564 was carried out in at least triplicate on each sample using a StepOnePlus real-time PCR system 565 (Applied Biosystems, UK) and Fast SYBR green master mix (Life Technologies, UK). Data were 566 analysed using Expression Suite Software version 1.1 (Life Technologies, UK) using fabD and rpoD as endogenous controls and taking primer efficiency into account. Changes in expression are in 567 568 comparison to the isogenic WT strain, where those exposed to 0.25 x MIC octenidine are compared 569 to WT exposed to 0.25 x MIC octenidine, and those not exposed are compared to WT not exposed. 570 Comparison of WT exposed and unexposed showed no significant changes in gene expression.

571 HR-MAS including for supernatant

572 Individual colonies of Pseudomonas aeruginosa strains selected from TSA plates were grown in 10ml 573 TSB, containing sub-inhibitory octenidine (¼ MIC) when applicable, in 50 mL centrifuge tubes at 37°C 574 overnight (~16 hours) without shaking. Optical density was measured by absorbance at 600nm to 575 ensure consistent growth to the stationary phase (OD ~1.2). The bacterial suspension was pelleted by centrifugation at 4000x g, 4°C for 10 minutes. The supernatant was sterilised by addition of 0.01% 576 (w/v) sodium azide and D₂O containing 2,2,3,3-D₄-3-(Trimethylsilyl) propionic acid sodium salt 577 578 $(TMSP-2,2,3,3-D_4)$ was added to a concentration of 10% (v/v) to provide a deuterium lock and 579 reference signal. The pellet was washed twice with PBS and snap frozen using liquid nitrogen and 580 dehydrated by freeze drying. Liquid state ¹H NMR spectra of spent media samples were acquired at 581 298 K and 600 MHz for ¹H on a Bruker Advance II 600 NMR spectrometer (Bruker Biospin, UK) with a 582 5 mm QCI helium-cooled cryoprobe and a refrigerated SampleJet sample changer to keep samples at 583 4°C prior to acquisition. 1D CPMG-presat (cpmgpr1d) experiments were acquired with 32 scans and 584 a spectral width of 19.8 ppm at a temperature of 298 K. 2D ¹H correlation spectroscopy (COSY) and ¹H-¹³C Heteronuclear Single Quantum Correlation (HSQC) experiments were performed on 585

586 representative data sets to assist metabolite assignment using Bruker settings. For HR-MAS NMR 587 Lyophilised pellets cell pellets were rehydrated with 40 μ l D₂O containing TMSP. Rehydrated pellets 588 were placed inside Kel-F inserts (Bruker, B4493) which were inserted into 4 mm Magic Angle 589 Spinning (MAS) rotors (Bruker, P/N H14355). All 1D spectra were recoded using the Bruker cpmgr1d 590 spin echo pulse with water presaturation delay of 1 second with a spectral width of 16.02 ppm and a 591 ¹H 90 pulse length was around 12 ms. HR-MAS NMR acquisition was carried out at 310 K with an 592 initial 8 dummy scans and 64 acquisition scans. The free induction decay was multiplied by an 593 exponential function with 0.293 Hz line broadening. Spectra were phased manually, and their 594 bassline corrected automatically using Topspin (Bruker). HR-MAS NMR 2D homonuclear shift correlation with presaturation during relaxation delay. ¹H COSY (cosyparf) and ¹H-¹³C correlation 595 596 HSQC (hsqcpc) spectra were also collected using standard Bruker pulse sequences to aid metabolite 597 assignment. All spectra were referenced to 0 ppm using the TSP reference peak. Metabolites were 598 assigned to NMR peaks using a combination of literature, the biological magnetic resonance data 599 bank (BMRB) metabolite database and Chenomx profiler software (Chenomx, Edmonton, Canada). 600 Assignments were confirmed using 2D 1 H COSY and natural abundance 13 C HSQC experiments. All 601 spectra were normalised using probabilistic quotient normalisation prior to analysis. Volcano and 602 box and whisker plots were created using the python modules numpy, pandas, scipy and seaborn. 603 Volcano plots provide a global view of all significant, relative changes in metabolites in a given 604 condition while box and whisker plots reveal the change in a particular metabolite across the full 605 range of isogenic mutants in octenidine unchallenged and challenged conditions. Fold change was 606 calculated by taking the ratio of peak intensities from the two sets of data being compared 607 (treatment/control). P values were calculated using the Mann-Whitney U test to compare means of 608 the treatment and control and a false rate was set using the Benjamini-Hochberg method (α = 0.05). 609 Partial least square regression was performed using the scikit learn python module using one 610 component. Data was split into 70/30 training/test sets and used for Monte Carlo cross-validation models. The procedure was run 1000 times to avoid bias by sample separation and model 611 performance was assessed through R^2 and Q^2 values (Supplementary Tables 13-14). Correlation 612 613 between metabolite intensities and fluorescence measurements for the same biological replicate 614 were assessed using the Spearman coefficient.

615 Laurdan/DPH

616 Pseudomonas aeruginosa strains were cultured as described for HR-MAS/spent media NMR 617 methods and bacterial suspension was fixed by addition of formaldehyde to a concentration of 618 0.25%. After fixation, cells were washed 3 times with PBS before resuspension in PBS for data 619 acquisition. Laurdan fluorescent dye or 1,6-diphenyl-hexa-1,3,5-triene (DPH) was added to a final 620 concentration of 2.5 µM and samples were incubated with either dye for one hour prior to 621 acquisition with minimal exposure to light. For acquisition, samples were placed in a quartz cuvette 622 and measurements taken using a Varian Cary Eclipse fluorescence spectrometer (Mulgrave, Victoria, 623 Australia). Laurdan fluorescence measurements were taken by excitation at 350 nm and emission 624 was detected between 400-600 nm. General polarisation was calculated using the following 625 equation where I_{440} and I_{490} stands for intensity at 440 nm and 490 nm respectively:

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

DPH anisotropy measurements were taken by excitation at 358 nm and emission was collected at430 nm. Anisotropy was calculated using the following equation:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} - 2GI_{VH}}$$

628 I_{VV} and I_{VH} are the parallel and perpendicular polarized fluorescence intensities measured with 629 vertically polarized excitation light. I_{HV} and I_{HH} are the same fluorescence intensities measured 630 horizontally polarised light. G is the monochromator grating correction factor given by G = I_{HV}/I_{HH} . 631 OriginPro version 8 was used to calculate statistical significance using a one-way ANOVA and the 632 Tukey-Kramer post-hoc test with a value of $p \le 0.05$ to establish statistical significance.

633 Microscopy and analysis

634A fixative solution was prepared comprising 2.5% glutaraldehyde, 0.1M PIPES buffer and 13.87 mM635glucose dissolved in ultrapure water. A wash buffer was prepared from the same reagents, excluding636the glutaraldehyde. Four strains were selected for transmission electron microscopy analysis: WT,637SmvR Δ106-109, PssA D240G and the double mutant SmvR Δ106-109 PssA D240G. Appropriate sub-638inhibitory concentrations were determined to be 0.25x MIC for the single mutants and 0.125x MIC639for the WT and double mutant.

640 Control and challenge samples were grown in 10 mL TSB or TSB with sub-inhibitory octenidine and 641 pelleted at 4°C. Pellets were then washed with fixative buffer and fixed in the same buffer overnight 642 at 4°C. Samples were osmicated in 1% osmium tetroxide in cacodylate buffer for 1.5 hours at 4°C 643 and then washed twice with distilled water. Samples were stained with 1% uranyl acetate in water 644 for 1 hour at room temperature and washed again in distilled water before dehydrating in a series of 645 ethanol. Pre-infiltration with propylene oxide was carried out twice, both for 10 minutes before 646 samples were used to infiltrate a series of SPURR resin:propylene oxide mixture followed by 647 infiltration of 100% SPURR resin for 24 hours. Samples were then embedded and polymerised for 24 648 hours at 60°C. Images were acquired using a Jeol 1400 plus microscope at 120 kV with a Jeol Ruby 649 camera (Jeol, USA).

- 650 The resulting images were analysed qualitatively as well as with an in-house MATLAB script
- 651 leveraging the Image Processing Toolbox. Images were processed to include only in-plane bacteria,
- and the major axis length (cell length) was recorded for each cell. Histograms of cell length with
- 653 fitted distributions were then generated using the MATLAB Statistics Toolbox.

654 Data Availability.

The datasets generated and analysed during the current study are available in the BioProject (SubmissionID: SUB6110485, BioProject ID: PRJNA558315) and Metabolights repository (www.ebi.ac.uk/metabolights/MTBLS1681). Source data is presented in the Supplementary materials. All other data are available from the corresponding authors on reasonable request.

660 **Tables**

661

662 <u>Table 1</u> Mutations present in *P. aeruginosa* strains passaged in increasing concentrations of

663 octenidine followed by 10 passages on TSA in the absence of octenidine. LCV large colony variant;

Parental	SmvR	PssA	PgsA	Other	OCT MIC fold increase
strain	(186aa)	(271aa)	(186aa)		over parental strain
NCTC 13437	K51R*	D240E		SNP in <i>smvA/R</i> promoter	256
372261 LCV	R3C	V222G			32
372261 SCV	123*			ParS A168V	32
CAS2	∆43-52	D240G		PmrB S284N, HasR V667I	128
CAS3	A143P	V222G			64
CAS4	Δ82-109		T58M	PmrB T132P	32
GH12	Δ106-109	V222G			64
PAO1	Δ106-109			PA3328 P51H (probable FAD-	2
				dependent monooxygenase)	

664 SCV small colony variant; aa amino acid; * premature stop codon

665

_

666 <u>Table 2: Minimum inhibitory concentrations (mg/L) of a selection of biocides and antibiotics for</u>

667	PAO1 WT and isogenic mutants (OCT – octenidine, CHD – chlorhexidine digluconate, ALE	ΞX –
-----	--------------------------------------------------------------------------------------	------

668	alexidine, DDAB – didecyldimethylammonium bromide, TOB – tobramycin, AMI – amikacin, CST –							
669	colistin)							
		ОСТ	CHD	ALEX	DDAB	тов	AMI	CST
WT		2	4	4	8	8	2	0.5

WT	2	4	4	8	8	2	0.5
PssA V222G	8	4	2	8	8	2	0.5
PssA D240G	8	4	4	8	8	2	0.25-0.5
PssA D240E	4	4	4	8	8-16	2	0.5
PgsA T58M	4	4	2	8	16	2	0.5
SmvR Δ106-109	4	8	4	8	8	2	0.5
PssA V222G SmvR Δ106-109	64	4	4	8	8	2	0.25-0.5
PssA D240G SmvR Δ106-109	64	4	4	8	8	2	0.25-0.5
PssA D240E SmvR Δ106-109	64	16	8-16	8	8	4	0.5
PgsA T58M SmvR Δ106-109	32	8	4	8	8-16	2	0.5

670 **Figure Legends**

671 Figure 1: The octenidine tolerance of strain populations increases with exposure to increasing 672 octenidine concentrations and is linked to mutations in SmvR and then PssA/PgsA. Seven strains of 673 P. aeruginosa were exposed to doubling concentrations of octenidine as shown on the x-axis and in 674 Supplementary Figure 2. Mutations in SmvR (black) occur at low octenidine concentrations and 675 result in low levels of increased octenidine tolerance. Deletion of amino acids 106-109 occurs in all 676 strains. Mutations in PssA (blue) and PgsA (purple) occur at higher octenidine concentrations and 677 result in higher octenidine tolerance. Mutations were identified using population-mode BreSeq 678 (P \leq 0.01) on PCR products of the genes amplified from populations. \geq 10% of the population is listed 679 in the figure, for % of the population containing specific mutations see Supplementary Table 4. n=1 680 experiment for each strain* premature stop codon; x2 duplication of listed amino acids; fs frame 681 shift following listed amino acid; final = single colony restreaked 10 times on TSA in the absence of 682 octenidine, for 372261 a large colony variant (R3C in SmvR and V222G in PssA) and a small colony 683 variant (123* in SmvR) were picked.

684 Figure 2: Mutations following octenidine adaptation lead to constitutively expressed *smvA*.

Expression levels of *smvA* (blue), *smvR* (yellow), *pssA* (green) and *pgsA* (red) in isogenic strains of *P*.

686 *aeruginosa* containing mutations in SmvR (Δ106-109), PssA (V222G, D240G, D240E) and PgsA

687 (T58M), individually or in combination, were compared to wild type expression levels. No significant

688 changes were detected for *pssA* or *pgsA* (a). Expression was also measured following exposure to

689 0.25 x MIC octenidine for 30 minutes compared to the wild type strain exposed to octenidine (b).

- Significant RQ values are shown (*** $P \le 0.01$, ** $P \le 0.05$, * $P \le 0.1$). Error bars represent RQ Min and
- RQ Max, data points represent means of three technical replicates. Complete results are shown inTables S9-S10.

693 Figure 3. Effect on *P. aeruginosa* PAO1 metabolism of isogenic mutations in the presence/absence 694 of octenidine. The major catabolic pathways of *P. aeruginosa* (a). PQN normalised H^1 NMR 695 resonance intensities, proportional to concentration, of metabolites in fresh or spent media after 696 growth of recombinant PA01 strains to the stationary phase (OD₆₀₀ ~1.2) (b-j). Exp denotes 697 octenidine exposure within growth medium at 0.25x MIC. For each condition a minimum of n=6 698 individual colonies were grown in 6 individual cultures. * shows significant differences with respect 699 to WT, as determined by one-way ANOVA with Tukey's post hoc test ($p \le 0.05$). For strains exposed to 700 octenidine (exp), significance is shown with respect to WT exp.

701 Figure 4. Membrane biophysical parameters in *P. aeruginosa* PAO1 and isogenic strains in the

presence/absence of octenidine stress. Measures of lipid order – Laurdan GP (a), and lipid fluidity –
 DPH fluorescence anisotropy (b) are shown for fluorescently labelled bacteria grown with or without
 0.25x MIC octenidine in TSB. Spearman correlations from a PLS regression model are shown

705 between Laurdan generalised-polarisation (GP) and ¹H HR-MAS NMR resonance intensity of

intracellular metabolites or cell envelope components, which is proportional to their concentration.

707 Recombinant PA01 strains were grown in TSB or TSB with 0.25x MIC octenidine to the stationary

- phase (OD₆₀₀ ~1.2). Each fluorescence and HR-MAS NMR measurement was carried out on the same
- 509 biological replicate with a minimum of n=6 biological replicates carried out for each condition. PLS
- regression models were validated by splitting data into 70/30 training/test sets for use with Monte
- 711 Carlo cross-validation models. The procedure was run 1000 times to avoid bias by sample separation
- and model performance was assessed through R2 and Q2 values (Supplementary Tables 13-14). Low
- 713 GP values correlate with increased lipid –CH₂ (f), lipid –CH₃ (Supplementary Figure 16) and glutamate

(d) and lower levels of acetate (c), ethanolamine (e) and signals from N-acetyl (4) (g) and glycerol in a
lipid headgroup (h).

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717 Figure 5. Schematic summary of results and hypotheses. 1 A low concentration (<MIC) of 718 octenidine enters the cell in which SmvR is bound to the promoter of smvAR; 2 Octenidine binds to 719 SmvR releasing smvAR promoter, smvR and smvA are increasingly transcribed, SmvR is bound by 720 octenidine, SmvA is inserted into the inner membrane; 3 SmvA effluxes octenidine into the 721 periplasmic space; 4 Octenidine is effluxed through the outer membrane by an unknown 722 mechanism, possibly by redundant pumps (e.g. OprJ, OprM, OprN, OmpD); 5 Mutations in smvR lead 723 to constitutive expression of SmvA, 6 Overexpressed SmvA also effluxes glycine-betaine cations 724 leading to oxidative stress, which destabilises the membrane; 7 At increased octenidine 725 concentrations (≥MIC) mutated PssA and PgsA modify the membrane, changing fluidity, order and 726 charge with the following possible consequences: more stable SmvA or support with folding; more 727 stable membrane under octenidine stress; less cation/octenidine binding or increased 728 cation/octenidine release; increased proton gradient supporting glyoxylate shunt, increased efflux 729 by SmvA and decreased oxidative stress; 8 Increased resistance to cationic antimicrobials may lead 730 to increased resistance to antimicrobial peptides

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