







Sex Steroids Induce Membrane Stress Responses and Virulence Properties in *Pseudomonas aeruginosa*

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ABSTRACT Estrogen, a major female sex steroid hormone, has been shown to promote the selection of mucoid *Pseudomonas aeruginosa* in the airways of patients with chronic respiratory diseases, including cystic fibrosis. This results in long-term persistence, poorer clinical outcomes, and limited therapeutic options. In this study, we demonstrate that at physiological concentrations, sex steroids, including testosterone and estradiol, induce membrane stress responses in *P. aeruginosa*. This is characterized by increased virulence and consequent inflammation and release of proinflammatory outer membrane vesicles promoting *in vivo* persistence of the bacteria. The steroid-induced *P. aeruginosa* response correlates with the molecular polarity of the hormones and membrane fluidic properties of the bacteria. This novel mechanism of interaction between sex steroids and *P. aeruginosa* explicates the reported increased disease severity observed in females with cystic fibrosis and provides evidence for the therapeutic potential of the modulation of sex steroids to achieve better clinical outcomes in patients with hormone-responsive strains.

IMPORTANCE Molecular mechanisms by which sex steroids interact with *P. aeruginosa* to modulate its virulence have yet to be reported. Our work provides the first characterization of a steroid-induced membrane stress mechanism promoting *P.*

Citation Vidaillac C, Yong VFL, Aschtgen M-S, Qu J, Yang S, Xu G, Seng ZJ, Brown AC, Ali MK, Jaggi TK, Sankaran J, Foo YH, Righetti F, Nedumaran AM, Mac Aogáin M, Roizman D, Richard J-A, Rogers TR, Toyofuku M, Luo D, Loh E, Wohland T, Czarny B, Horvat JC, Hansbro PM, Yang L, Li L, Normark S, Henriques-Normark B, Chotirmall SH. 2020. Sex steroids induce membrane stress responses and virulence properties in *Pseudomonas aeruginosa*. *mBio* 11:e01774-20. <https://doi.org/10.1128/mBio.01774-20>.

Editor Vanessa Sperandio, University of Texas Southwestern Medical Center Dallas

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Received 28 June 2020

Accepted 26 August 2020

Published 29 September 2020

Changes made 27 October 2020

aeruginosa virulence, which includes the release of proinflammatory outer membrane vesicles, resulting in inflammation, host tissue damage, and reduced bacterial clearance. We further demonstrate that at nanomolar (physiological) concentrations, male and female sex steroids promote virulence in clinical strains of *P. aeruginosa* based on their dynamic membrane fluidic properties. This work provides, for the first-time, mechanistic insight to better understand and predict the *P. aeruginosa* related response to sex steroids and explain the interindividual patient variability observed in respiratory diseases such as cystic fibrosis that are complicated by gender differences and chronic *P. aeruginosa* infection.

KEYWORDS hormones, steroids, *Pseudomonas aeruginosa*, membrane stress, gender

Clinical observations and research reveal that males are at higher risk for bacterial infection; however, females are more prone to develop more severe illness with poorer outcomes, particularly at reproductive age (1–3). Genetic, anatomical, social, and behavioral differences have all been advanced to explain these gender biases, however, more recently, variations in the host innate and adaptive immune responses under the control of sex steroid hormones have been proposed (4–6). Estrogens exert immunoprotection by promoting natural killer cell activity and inducing the production of proinflammatory cytokines, including tumor necrosis factor alpha (TNF- α), interleukin-1 (IL-1), IL-6, IL-17, and IL-23, while testosterone exerts an opposing immunosuppressive effect (5). The regulation of immune responses by sex steroid hormones may at least partially explain differences in disease susceptibility; however, it does not fully explain the observed differences in disease severity and, importantly, microbial adaptation that occur between genders in the context of chronic pulmonary infection (7).

The last 2 decades have witnessed an exponential rise in the number of studies investigating the role of sex steroids on the progression of chronic infections (7–13). Many bacterial pathogens sense and respond to mammalian hormones, including sex steroids (5, 8, 13, 14). Nanomolar physiological concentrations of 17 β -estradiol and estriol promote *Pseudomonas aeruginosa* alginate and pyocyanin production, surface motility, biofilm formation, and adherence to lung epithelia (8, 12). In contrast, micromolar supraphysiological concentrations of 17 β -estradiol and testosterone inhibit quorum sensing (14). The influence of sex steroids on clinical disease is exemplified in the genetic condition cystic fibrosis (CF), where elevated 17 β -estradiol correlates with the emergence of the deleterious mucoid phenotype of *P. aeruginosa*, a variant associated with poorer lung function and increased mortality, suggesting a key role for sex steroids on the bacterial pathogenicity (8). Despite these links, the mechanisms that underpin the specific roles of sex steroids on bacterial physiology and pathogenicity remain unknown. The presence of receptor proteins, such as LasR in *P. aeruginosa*, TraR in *Escherichia coli*, and even some ubiquitous efflux transporters such as AcrAB-TolC or ErmAB-TolC, that may recognize mammalian sex hormones has been suggested; however, no binding interactions or molecular mechanisms leading to virulent bacterial phenotypes are reported (5, 14–17).

Here, we provide evidence, using both *in vitro* and *in vivo* models, that natural and synthetic sex steroid hormones modulate *P. aeruginosa* disease-causing capabilities by inducing bacterial membrane stress responses and production of outer membrane vesicles (OMVs). The steroid concentration required to induce these bacterial responses inversely correlates with the hydrophobic nature of the compound. We propose that clinical isolates may be categorized as hormone-responsive or not depending on their membrane fluidic properties, which bears therapeutic potential in the treatment of *P. aeruginosa* chronic lung infections. This work offers fresh insight into our understanding of sex differences in the susceptibility and response to infections caused by *P. aeruginosa* in the clinic and highlights the potential impact of nonantimicrobial stressors on infectious disease outcomes.

RESULTS

Sex steroid hormones promote the virulence of *P. aeruginosa*. The female sex steroid hormone estrogen has been known to induce alginate production by *P. aeruginosa*, which is crucial for the mucoid conversion and survival of the bacteria in the human host (8, 14). Thus, we first assessed the ability of four different sex steroid hormones to alter *P. aeruginosa* alginate production. The tested compounds varied in polarity and included the synthetic hormone ethinylestradiol (EE2) (logP, 4.15) (18) (least polar), testosterone (T) (logP, 3.30) (19), the pregnancy-related estrogen estriol (E3) (logP, 2.45) (20), and a water-soluble synthetic and hydrophilic derivative of testosterone, TSDG.HCl (logP, 2.40) (21) (most polar) (Fig. 1A). Using a range of concentrations varying from 250 pM to 250 μ M, we observed that while all compounds induced *P. aeruginosa* PAO1 alginate production, the concentration required to achieve the maximal response for each compound decreased as their molecular polarity increased (i.e., hydrophobicity decreased) (Fig. 1B). Thus, the most polar compounds, TSDG.HCl and estriol, required concentrations of \approx 25 nM to significantly increase PAO1 alginate production, while molecules with lower polarity, testosterone and EE2, required 2.5 μ M and 250 μ M, respectively. Their effect was further confirmed by an increased swarming motility and rhamnolipid and elastase production, three major virulence factors produced by *P. aeruginosa* (Fig. 1C to E and Fig. S1A-C). The responses found were not attributable to differences in inocula or bacterial growth rates, as results were normalized at the time of cell harvest (see methods), and growth kinetics did not show any significant differences when *P. aeruginosa* was cultured with the different steroid compounds (Fig. S1D). Since it has been suggested that swarming motility has an impact on biofilm formation (22, 23), we next studied *P. aeruginosa* PAO1 biofilms grown in a hormone-rich environment. We observed a significantly reduced roughness coefficient of biofilms grown in the presence of testosterone (2.5 μ M) or estriol (25 nM), illustrating a flat homogeneous architecture of the bacteria compared to vehicle control (ethanol) (Fig. 1F to G). Changes in the biofilm 3-dimensional morphology were not associated with differences in growth rates, as biofilms grown in the presence of sex steroids showed comparable numbers of viable biofilm embedded cells compared to ethanol (vehicle control) (Fig. 1H). However, biofilms grown in the presence of hormones had significantly increased biovolumes, a finding that is consistent with the increased alginate production found earlier (Fig. 1B and G). This shift in biofilm architecture further translated into an altered matrix and its related diffusion properties when tested with the neutral dextran molecule (150 kDa) that showed a significantly decreased molecular diffusion across PAO1 biofilms grown in the presence of testosterone compared to ethanol (vehicle control) ($1.30 \pm 0.97 \mu\text{m}^2/\text{s}$ versus $2.68 \pm 1.82 \mu\text{m}^2/\text{s}$, respectively; $P < 0.05$) (Fig. 1I).

To study the effects of steroid hormones *in vivo*, we used mouse models of lung infection where PAO1 bacteria were inoculated intranasally. By comparing PAO1 pre-exposed to ethanol (vehicle control) with treatment with the respective hormones (testosterone at 2.5 μ M and estriol at 25 nM), we observed a significant increase in bacterial counts (CFU) in the lungs of mice treated with testosterone ($P < 0.0001$) and a trend toward significance with estriol (Fig. 1J and K). This occurred even though it was a short-term experiment (6 h) and *P. aeruginosa* is not a natural pathogen and typically results in variable infections. These data are consistent with our *in vitro* observations, suggesting an *in vivo* persistence due to sex steroid exposure. We did not detect any significant inflammatory changes in the bronchoalveolar lavage fluid over this time period (Fig. S2).

Sex steroids promote *P. aeruginosa* virulence through the *muc* operon. Since our data demonstrate that steroid treatment of *P. aeruginosa* affects its virulence mechanisms, we next used transcriptomic analyses of the bacteria to identify the potential molecular mechanism(s) that drive these effects. Exposure of PAO1 to testosterone (Fig. 2A) led to major transcriptomic alterations (measured as fold change [FC] expression > 1.5) in key virulence genes involved in surface sensing (c-di-GMP

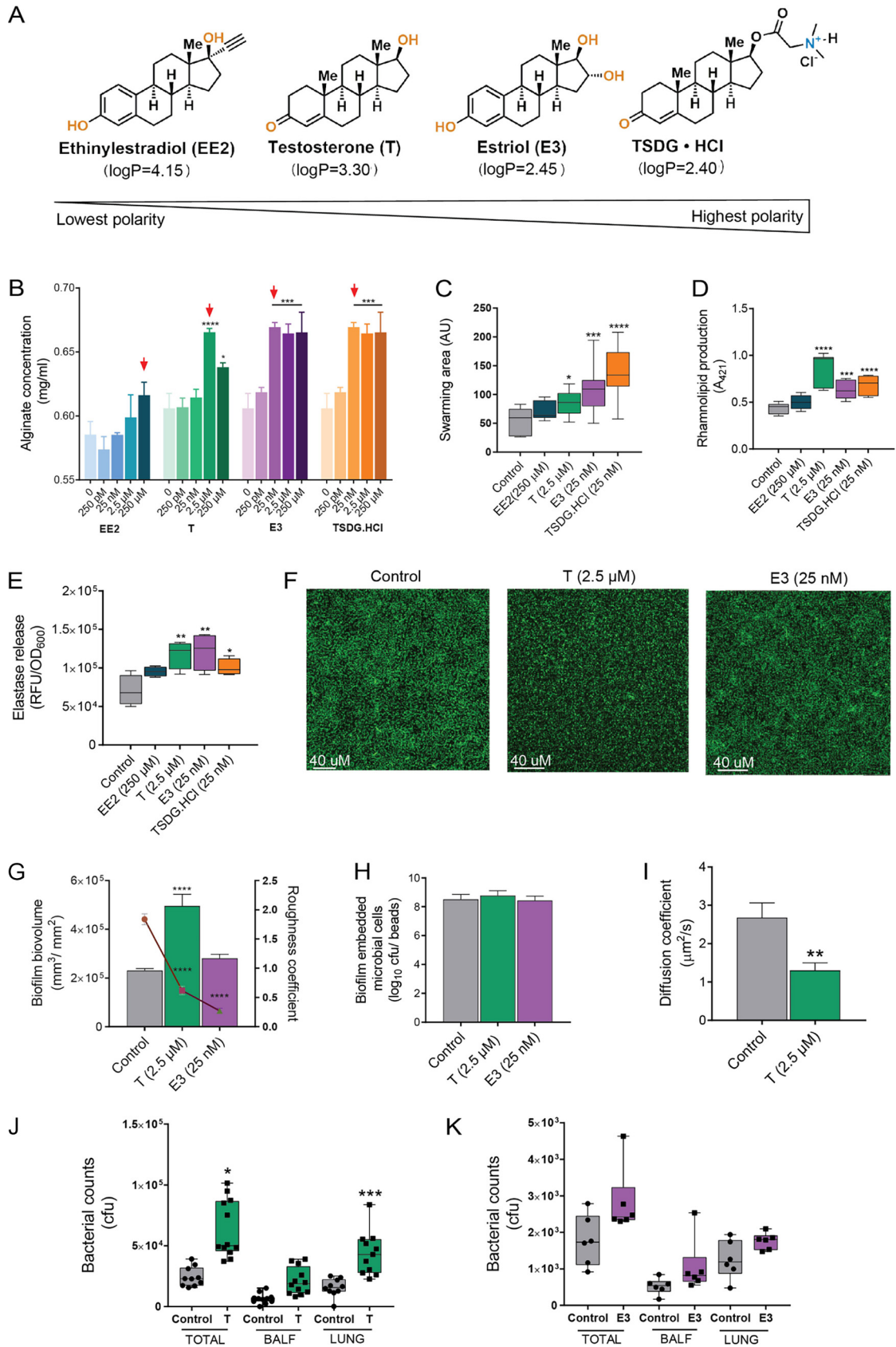


FIG 1 Sex steroids promote *Pseudomonas aeruginosa* virulence *in vitro* and reduce lung clearance *in vivo*. (A) Chemical structures of the various sex steroid hormones with associated logP values. (B) *P. aeruginosa* PAO1 was cultured for 16 to 24 h in tryptic soy broth (Continued on next page)

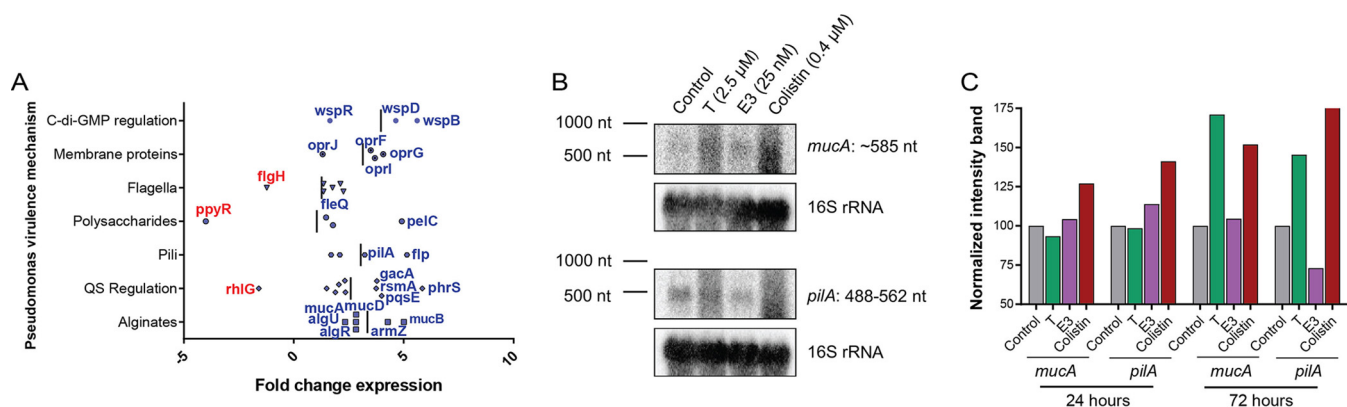


FIG 2 The sex steroids testosterone and estradiol induce *Pseudomonas aeruginosa* virulence through molecular signaling pathways controlled by surface sensing and membrane stress. (A) *P. aeruginosa* PAO1 was cultured for 16 to 24 h in tryptic soy broth (TSB) medium supplemented with ethanol (vehicle control) or testosterone (2.5 μM) and subjected to RNA sequencing. Significant increases in key virulence genes (marked in blue) involved in surface sensing (c-di-GMP regulation, pili), membrane stress (polysaccharides, alginates, quorum sensing, membrane proteins), and motility (flagella) illustrate the global regulation driven by testosterone. Three genes involved in flagellar motility, rhamnolipids, and polysaccharide regulation were found to be significantly downregulated (marked in red). All the genes reported with a significant change in expression (defined as a >0.5-fold increase or decrease compared to the control) are represented. (B and C) *P. aeruginosa* PAO1 was cultured for 24 to 72 h in TSB medium supplemented with ethanol (vehicle control), testosterone (2.5 μM), estradiol (25 nM), or colistin (0.4 μM, 1/4 MIC) as a positive control for the membrane stress response. Northern blot gels (B) show increased *mucA* and *pilA* mRNA expression following testosterone, estradiol, and colistin exposure, further illustrated by their (C) corresponding normalized band intensities. T, testosterone; E3, estradiol.

regulation and pili), membrane stress (polysaccharides, alginates, quorum sensing, and membrane proteins), and motility (flagella). These results suggest that global regulatory mechanisms that are activated by molecular signaling pathways are induced by *P. aeruginosa* envelope stress upon sex steroid exposure. Using colistin, a known membrane stress inducer, as a positive control (24), Northern blot analysis of PAO1 confirmed this (Fig. 2B and C). Thus, following testosterone (2.5 μM), estradiol (25 nM), and colistin (0.4 μM) treatments, we observed increased expressions of *mucA*, a gene encoding an anti-sigma factor that controls alginate biosynthesis and mucoid conversion (25), and *pilA*, a gene encoding pilin, the main constituent of type IV pili, key for surface motility, cell adhesion, and biofilm formation (26).

To identify proteins that bind to steroids, we next performed coimmunoprecipitation assays of testosterone and 17β-estradiol with *P. aeruginosa* lysates combined with mass spectrometry (MS) analysis. This revealed 11 and >50 protein hits for testosterone and 17β-estradiol, respectively (Table S1A). The *P. aeruginosa* master regulator of virulence, Vfr, was identified as a testosterone-associated protein and therefore subjected to further analysis. In addition to assessing the impact of steroid exposure on *P. aeruginosa* lacking *vfr* (Fig. S3), further evaluation of protein-ligand interactions was

FIG 1 Legend (Continued)

(TSB) medium supplemented with ethanol (vehicle control) or the respective sex steroid hormones at concentrations varying from 250 pM to 250 μM, and alginate production was assessed. Sex steroids significantly promote *P. aeruginosa* *in vitro* production of alginates in a concentration-dependent manner with an inverse relationship with molecular polarity. (C to I) *P. aeruginosa* PAO1 was cultured for 16 to 24 h in TSB medium supplemented with ethanol (vehicle control) and the respective sex steroid hormones (at optimal concentrations varying from 25 nM to 250 μM). Changes in virulence were assessed by (C) increased swarming motility, (D) increased rhamnolipid production, (E) elevated elastase levels, and (F) altered biofilm architecture with (G) reduced roughness coefficient and increased biovolumes, (H) unchanged microbial cell burden, and (I) reduced diffusivity for the neutral molecule of dextran (150 kDa) in the matrix. Each experiment was performed in triplicate, and results are presented as the geometric mean and standard error (B, G to I) or box and whisker plots with median (horizontal bar), interquartile range (boxes), and minimum and maximum (whiskers) (C to E). Statistical significance was determined by one-way ANOVA corrected using Dunnett's test for multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (J and K) *P. aeruginosa* PAO1 was cultured for 24 h in Luria-Bertani medium supplemented with ethanol (vehicle control), testosterone (2.5 μM), or estradiol (25 nM). Then, 10^6 CFU were used to infect the lungs of BALB/c female mice (6 to 10 weeks old) intranasally. Bacterial cell burden measured at 6 h postinoculation in bronchoalveolar lavage fluid (BALF) revealed a significantly reduced *P. aeruginosa* lung clearance with (J) testosterone (2.5 μM) and a trend toward reduced clearance with (K) estradiol (25 nM). Each experiment was performed in duplicate, and results are presented as box and whisker plots with median (horizontal bar), interquartile range (boxes), and minimum and maximum (whiskers). Statistical significance was determined by one-way ANOVA corrected using Tukey's test for multiple comparisons. *, $P < 0.05$; ***, $P < 0.001$. EE2, ethinylestradiol; T, testosterone; E3, estradiol; TSDG.HCl, 17β-N,N-dimethylglycinate testosterone hydrochloride; AU, arbitrary unit; RFU, relative fluorescence unit.

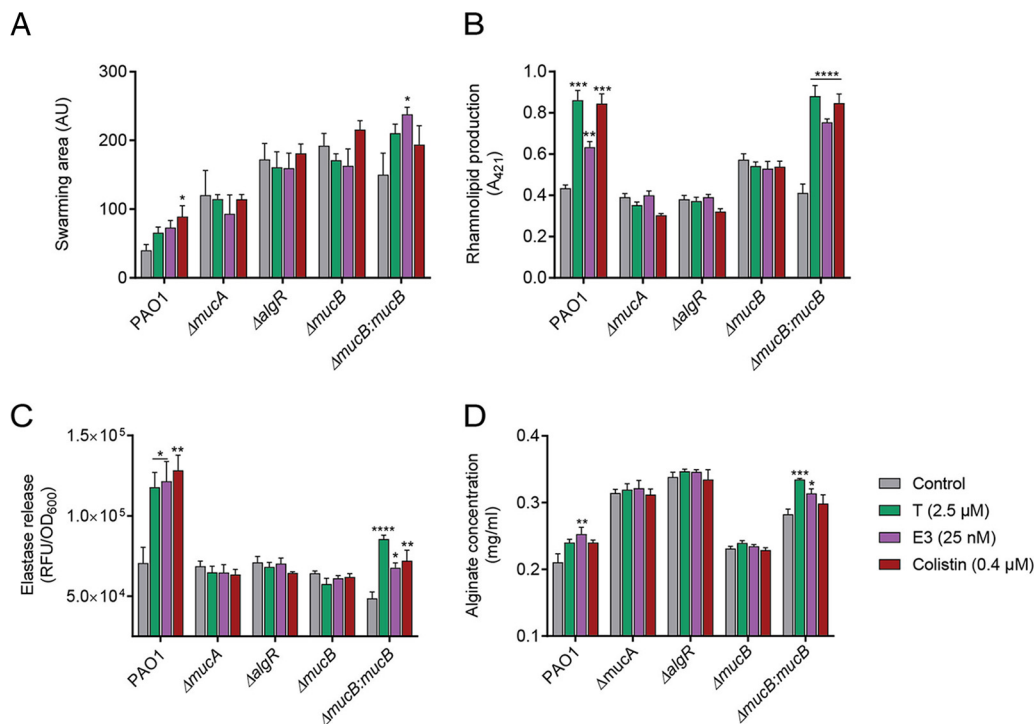


FIG 3 The sex steroid hormones testosterone and estriol induce enhanced *Pseudomonas aeruginosa* motility, rhamnolipid, elastase, and alginate production through the *muc* operon. (A) *P. aeruginosa* PAO1 and mutants lacking genes from the *muc* operon (*algR*, *mucA*, and *mucB*) were cultured for 24 h in tryptic soy broth medium supplemented with ethanol (vehicle control), testosterone (2.5 μ M), estriol (25 nM), or colistin (0.4 μ M, 1/4 MIC), and virulence was assessed. Testosterone and estriol induced increases in (A) swarming motility, (B) rhamnolipids, (C) elastase production in PAO1 but not in the *mucA*, *mucB*, or *algR* mutants, and the phenotype was restored following *mucB* complementation. Each experiment was performed in triplicate, and results are presented as the geometric mean and standard error. Statistical significance was determined by one-way ANOVA corrected using Dunnett's test for multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. T, testosterone; E3, estriol; AU, arbitrary unit; RFU, relative fluorescence unit.

performed using surface plasmon resonance (SPR). No direct interaction between Vfr and the main steroid hormones was found, suggesting that the sex hormones do not act directly on Vfr, but rather, act through a molecular pathway upstream of the *vfr* regulatory network (Fig. S4). Further analyses of the MS results identified *mucB* as a potential candidate. The *mucABCD* operon has been shown to influence *P. aeruginosa* virulence, mucoid conversion, and host persistence during chronic infections (27) (Table S1A). Analysis of *mucB* and steroid binding using SPR revealed no direct interaction (Fig. S4). We thus explored if the observed effects of steroids on *P. aeruginosa* virulence may be due to disruption of *mucB*-related signaling pathways. We exposed PAO1, its *mucB* mutant knockout derivative, and a *mucB* complemented strain to ethanol (vehicle control), testosterone (2.5 μ M), estriol (25 nM), and colistin (0.4 μ M) and assessed swarming motility, rhamnolipids, elastase, and alginate production (Fig. 3A to D). Interestingly, in the absence of *mucB*, PAO1 did not respond to testosterone, an effect that was restored by *mucB* complementation. To further confirm this hypothesis, we then investigated the role of other related genes in the *muc* operon and alginate biosynthetic pathways, *mucA* and *algR* (Fig. 3A to D). Using a PAO1 mutant knockout in *mucA* or *algR*, we observed that both steroids and colistin induced significant increases in swarming motility, rhamnolipids, elastase, and alginate production in wild-type (WT) PAO1 but not in the mutants, confirming that the effects of sex steroid exposure observed on *P. aeruginosa* occur through the *muc* operon.

Sex steroids induce *P. aeruginosa* membrane stress and production of outer membrane vesicles. *P. aeruginosa*, like many other Gram-negative bacteria, secretes OMVs in response to environmental stresses (28–30). We therefore evaluated the

CHARACTERISATION OF OUTER MEMBRANE VESICLES

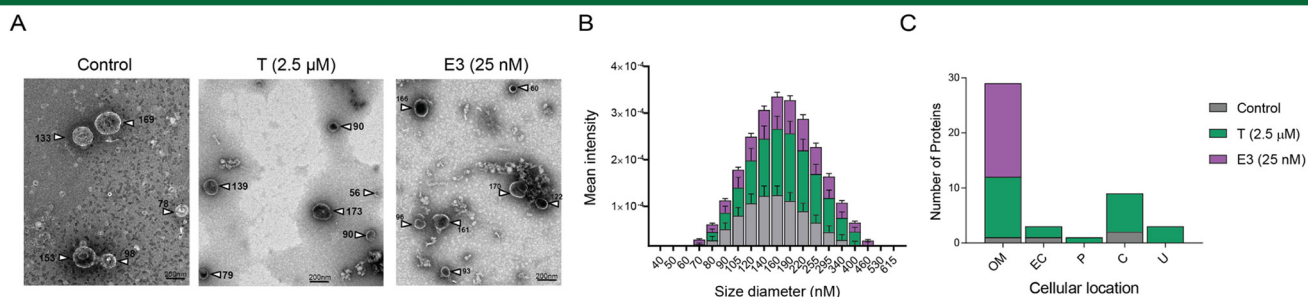
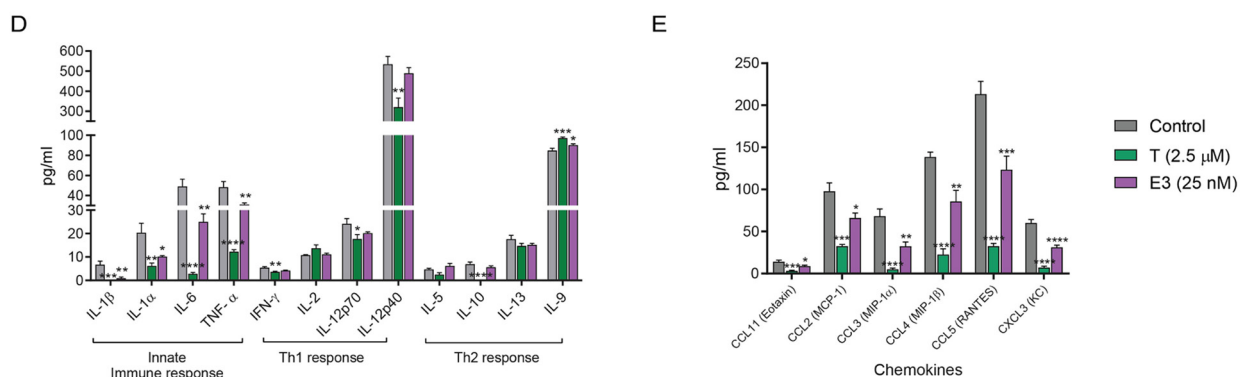
INFLAMMATORY EFFECT OF OUTER MEMBRANE VESICLES *IN VIVO*

FIG 4 The sex steroid hormones testosterone and estradiol induce release of *Pseudomonas aeruginosa* outer membrane vesicles with diminished inflammatory capability. *P. aeruginosa* PAO1 was cultured for 24 h in Tryptic soy broth medium supplemented with ethanol (vehicle control), testosterone (2.5 μM), or estradiol (25 nM). Outer membrane vesicles (OMVs) were extracted from the resulting supernatant and further purified as described in Materials and Methods. (A) Representative electron photomicrographs of extracted OMVs from cultures exposed to ethanol (vehicle control), testosterone (2.5 μM), or estradiol (25 nM) (arrows indicate OMVs with their respective vesicle size in nm). (B) OMV amount, but not size, differs under sex steroid stress (range, 80 to 400 nm). (C) OMV extracts subjected to proteomic analysis (Table S2B) reveal significant differences in protein composition and diversity and consist of predominantly outer membrane (OM) proteins. (D and E) Extracted OMVs (10 ng total protein) were administered to the lungs of female C57BL/6 mice ($n = 5, 6$ to 8 weeks old) using the intratracheal route. After 24 h, bronchoalveolar lavage fluid was obtained, and inflammatory (D) cytokines and (E) chemokines were measured, which illustrate a significantly diminished inflammatory response from OMVs secreted under sex steroid stress. Each experiment was performed in triplicate, and results are presented as the geometric mean and standard error. Statistical significance was determined by one-way ANOVA corrected using Dunnett's test for multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. T, testosterone; E3, estradiol; OM, outer membrane; EC, extracellular; P, periplasm; C, cytoplasm; U, unknown.

impact of sex steroid exposure (estradiol and testosterone) on OMV production. We found that *P. aeruginosa* PAO1 produces significantly more OMVs (2 to 3 times as many) in response to steroids (at their optimal concentrations) compared to the control (ethanol) (Fig. 4A and B). The size distribution of the secreted vesicles (80 to 400 nm) remained unchanged, but there were major differences in their protein composition. Proteomic analysis of purified OMV extracts exposed to sex steroids revealed a diverse array of predominantly outer membrane proteins, with significant alterations of the outer membrane lipid/protein composition and changes to cell envelope fitness compared to the control (Fig. 4C and Table S1B). While some proteins, including OmpA and flagellin, were detected in the OMVs produced under both control (ethanol) conditions and treatment with steroids, lipoproteins (PA2853, PA4423), efflux transporters, and type 6 secretion systems (T6SS) were more prominent after hormone exposure, suggesting that steroids induce alterations of the bacterial envelope (Table S1B). Interestingly, OMVs secreted during hormonal stress carried an uncharacterized protein, PA2462. This protein, previously identified in *P. aeruginosa* OMVs (31), also shares high sequence homologies with a filamentous hemagglutinin protein described in *Bordetella pertussis* that has been associated with bacterial adherence to ciliated eukaryotic cells (32). Next, we evaluated the *in vivo* effects of these OMVs by instilling purified extracts intratracheally in a model using C57BL/6 female mice (6 to 8 weeks old). Lung tissue and

bronchoalveolar lavage fluid (BALF) harvested at 24 h postinoculation illustrated the inflammatory response triggered by *P. aeruginosa* OMVs (Fig. 4D and E). Challenge with OMVs isolated during hormone treatment induced significantly lower cytokine (IL-1 α , IL-1 β , IL-6, and TNF- α) and chemokine (CCL2-5 and CXCL3) responses in the BALF 24 h postinfection than the control ($P < 0.001$). Lung histology correlated with these observations, and the pulmonary damage was most prominent after challenge with estriol-derived OMVs (Fig. S5). Taken together, our *in vitro* and *in vivo* data provide strong evidence that sex steroid hormones affect the disease-causing capability of *P. aeruginosa*.

Variation in membrane fluidity among *P. aeruginosa* clinical isolates explains sex-hormone responsiveness. To study the clinical relevance of our findings and determine if the effect of sex steroid hormones on *P. aeruginosa* virulence differs between clinical strains due to differences in their membrane fluidity, we performed fluorescence recovery after photobleaching (FRAP) studies. This technique is widely used to measure the mobility and interaction of fluorescently labeled biological molecules within living membranes. As a first measurement, we used the WT PAO1 strain, as well as the *mucB* mutant and its complemented derivative. Since the *muc* operon plays a central role in the response to membrane stress and production of OMVs (33), we speculated that mutations in this operon will translate into significant alteration of membrane fluidity. Consistent with this hypothesis, we found that the *mucB* mutant had a significant reduction in the fraction of recovery compared to PAO1, which was restored to wild-type levels in the *mucB* complemented strain (Fig. S6). Next, we assessed the recovery fractions of nine clinical isolates of *P. aeruginosa* isolated from different patients (Table S2A). Four of the nine isolates (44%) exhibited lower fractions of recovery compared to WT PAO1, two of which were statistically significant (patient isolates 1 and 2) (Fig. 5A). The five remaining isolates exhibited a higher fraction of recovery than WT PAO1 (patient isolates 5 to 9), two of which were statistically significant (patient isolates 8 and 9) (Fig. 5A). To test whether there was a correlation between the fraction of recovery (membrane fluidity) and steroid “susceptibility” of the isolates, we next assessed the impact of adding testosterone (at 25 nM and 2.5 μ M) on their swarming motility (Fig. 5B). As expected, we found that the isolates with a higher fraction of recovery, except isolate 8, showed a significant increased swarming capacity, while those isolates with a lower fraction of recovery were nonresponsive to steroid treatment. To further confirm the FRAP findings, we assessed two clinical isolates (5 and 9) against a larger panel of *P. aeruginosa* virulence factors. We found that both strains had enhanced rhamnolipids, elastase, and alginate production in the presence of testosterone or estriol at concentrations ranging from 25 nM (physiological) to 2.5 μ M (supraphysiological) (Fig. 5C to E). These data support that clinical isolates of *P. aeruginosa* respond differently to sex hormones depending on their membrane properties, which affects virulence.

DISCUSSION

The present study provides molecular and cellular evidence that sex steroid hormones modulate *P. aeruginosa* virulence through membrane stress and release of OMVs, which depends on bacterial membrane properties. In agreement with published data, we illustrate that estrogens induce alginate production (8, 14). However, we also show that a range of sex steroids, including male, female, natural, and synthetic hormones, have the potential to modulate *P. aeruginosa* pathogenicity by promoting surface motility, rhamnolipids, elastase, and alginate production and formation of structurally different biofilms with significantly reduced permeability (14, 22, 34, 35). The effects of the steroids correlate with their molecular polarity, a physicochemical property dictating their ability to diffuse across bacterial membranes (36–38). With higher molecular polarity, the diffusivity becomes slower through the bacterial membrane because of increased interactions with proteins, lipids, and polysaccharides, which in turn, increases the membrane stress. Also, the required steroid concentration to induce membrane stress is lower with higher polarity. Thus, the polarity of the

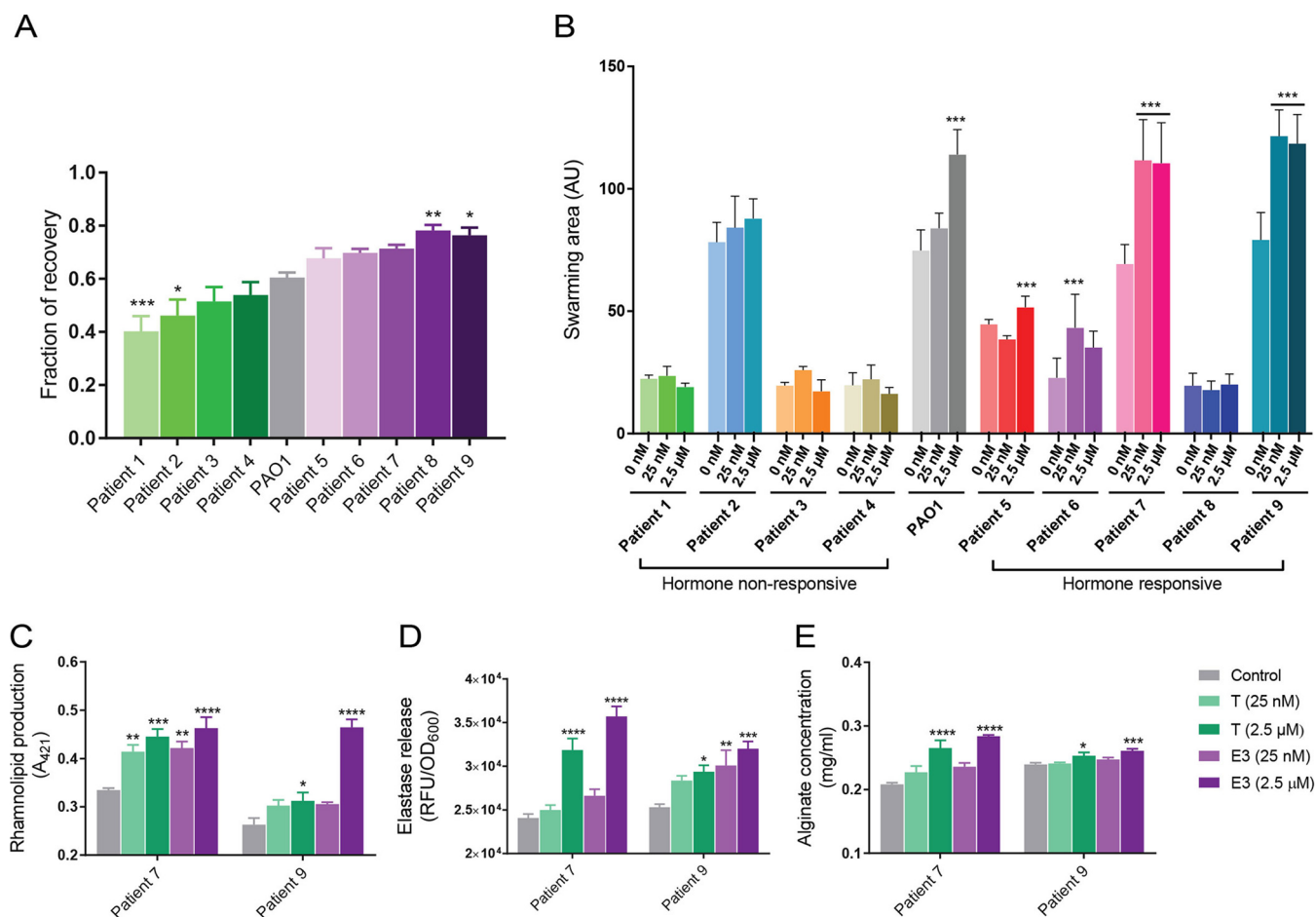


FIG 5 Variations in membrane fluidity among *Pseudomonas aeruginosa* clinical isolates explains sex hormone responsiveness. (A and B) Membrane fluidity (assessed using fluorescence recovery after photobleaching [FRAP]) of nine clinical *P. aeruginosa* isolates from nine patients was compared to PAO1. Four of the nine isolates (*P. aeruginosa* from patients 1 to 4) demonstrate lower membrane fluidity, while the other five (*P. aeruginosa* from patients 5 to 9) show higher membrane fluidity compared to PAO1. (B) Clinical *P. aeruginosa* isolates were cultured for 24 h in tryptic soy broth (TSB) medium supplemented with ethanol (vehicle control) or testosterone at two concentrations (physiological concentration, 25 nM; supraphysiological but optimal concentration for PAO1, 2.5 μM). The motility response (swarming) was assessed as an indicator of the sex steroid virulence response. The four isolates with the lowest membrane fluidity compared to PAO1 (*P. aeruginosa* from patients 1 to 4) did not demonstrate any motility changes following testosterone exposure (termed “nonresponsive”). In contrast, four of the five isolates (*P. aeruginosa* from patients 5 to 7 and patient 9) demonstrated significantly increased motility (and higher membrane fluidity) compared to PAO1 following testosterone exposure (termed “hormone-responsive”). (C to E) Two hormone-responsive clinical isolates (patients 7 and 9) were selected and assessed for further virulence studies. Strains were cultured for 24 h in TSB medium supplemented with ethanol (vehicle control), testosterone, or estriol, each at two separate concentrations (25 nM and 2.5 μM as described above). Both hormone-responsive strains exhibited significant upregulation of virulence markers upon hormone exposure as evidenced by elevated (C) rhamnolipids, (D) elastase, and (E) alginate production. Each experiment was performed in triplicate, and results are presented as the geometric mean and standard error. Statistical significance was determined by one-way ANOVA corrected using Dunnett’s test for multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. T, testosterone; E3, estriol; AU, arbitrary unit; RFU, relative fluorescence unit.

substance affects the *P. aeruginosa* membrane composition and integrity (39). Indeed, the most polar of the natural steroids, the pregnancy-related estrogen estriol required only nanomolar concentrations to alter *P. aeruginosa* virulence. These concentrations are physiological and have been previously demonstrated in bronchoalveolar lavage fluid (8). Although estriol is the predominant estrogen in pregnancy, it may also be detected in the lung following metabolism of estradiol by aromatase enzymes present in the airway epithelium (8, 40). In contrast, the least polar of all tested steroids, the synthetic estrogen used in oral contraception, ethinylestradiol, necessitated concentrations 1,000-fold higher than estriol to induce similar effects.

Furthermore, we found that the steroid-induced *P. aeruginosa* membrane change activates a global stress response controlled by the *muc* operon. This operon controls genes involved in *Pseudomonas* virulence such as surface motility (flagella function), biofilm formation, regulation of quorum sensing (*rhl*, *pqs*, and *las*), alginate biosynthe-

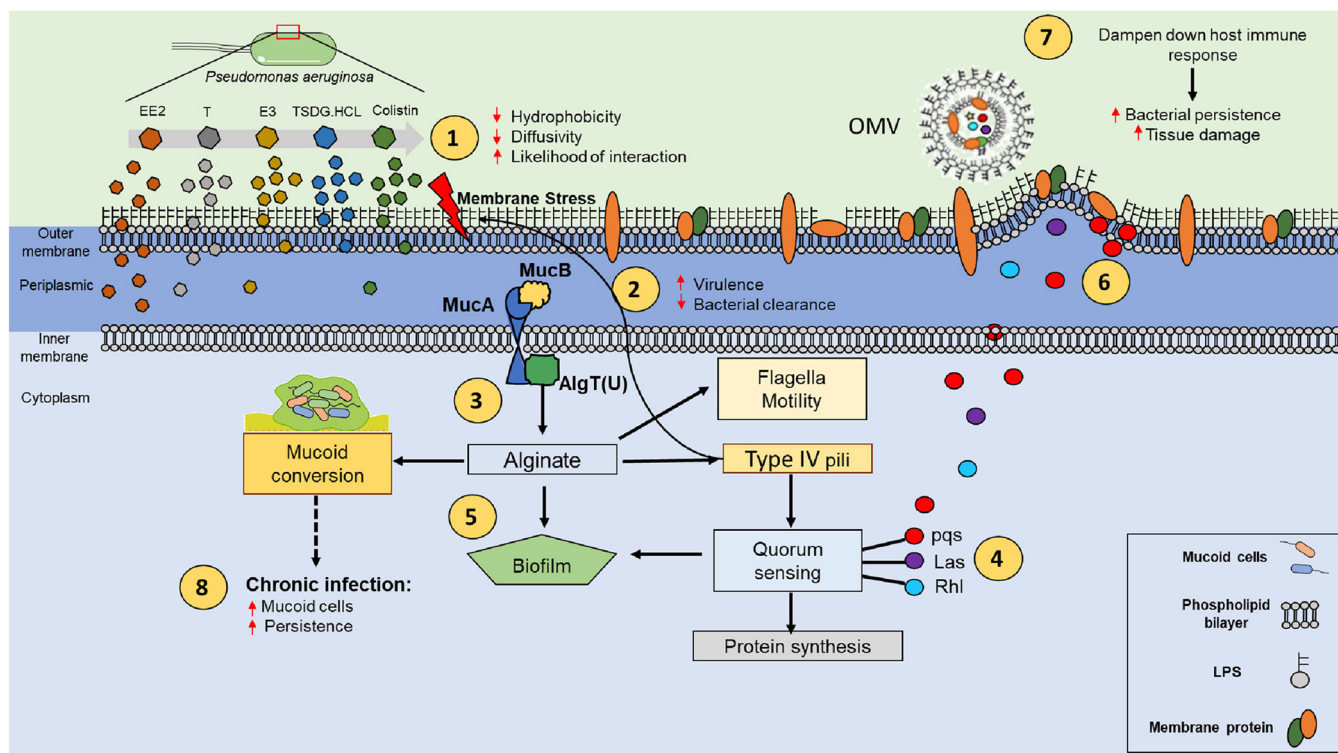


FIG 6 Proposed mechanistic model for sex steroid-driven regulation of *Pseudomonas aeruginosa* virulence and persistence. (1) The polarity of the various sex steroid hormones influences their ability to diffuse across the *P. aeruginosa* (lipid bilayer) membrane with their degree of impact on membrane dynamics dependent on the diffusion rate. More polar molecules such as estriol (with lower hydrophobicity) diffuse more slowly and therefore are more likely to interact with lipopolysaccharides and proteins in the bacterial cell envelope compared to less polar molecules such as testosterone or ethinylestradiol (with higher hydrophobicity), which diffuse more rapidly through the bacterial membrane. (2) Interactions between polar sex steroids, membrane proteins, and lipopolysaccharides are sensed by surface signaling systems, including the *muc* operon (*mucA/B*), leading to (3) activation of the alginate biosynthetic pathway through the *muc* operon, (4) upregulation of downstream genes involved in surface sensing (pili, c-di-GMP) and quorum sensing (*pqs*, *las*, *rhl*, *vfr*), and (5) biofilm formation (polysaccharides, alginates). (6) The increased secretion of quorum sensing autoinducer (AI) molecules coupled to increased membrane protein production alters membrane composition and fluidity and promotes “lipid raft” formation and membrane stress-inducing (7) OMV release that dampens host inflammatory responses and further promotes bacterial persistence and tissue damage. (8) In chronic *P. aeruginosa* infection with “hormone-responsive” strains, the constant pressure exerted by sex steroids, particularly on the bacterial envelope, promotes adaptive change, which aims to protect the bacterium by creating defensive barriers such as alginates that surround bacterial cells, leading to mucoid conversion with bacterial persistence and poorer clinical outcomes. EE2, ethinylestradiol; T, testosterone; E3, estriol; TSDG.HCl, 17 β -N,N-dimethylglycinate testosterone hydrochloride; OMV, outer membrane vesicle; LPS, lipopolysaccharides.

sis, and type IV pili (41). Activation of quorum sensing contributes to shaping the bacterial community, including a switch to biofilms and inter- and intraspecies communication through the diffusion of autoinducer (AI) molecules (*pqs* and *las*). Increased production of AIs and alginate alters the outer membrane properties and promotes lipid raft formation and OMV release (33). Indeed, we observed increased production of OMVs in response to steroids. OMVs affect bacterial persistence and host immune responses and tissue damage (42). OMV release following sex steroid exposure represents an underappreciated and important bacterial virulence mechanism (28, 43). With a central role in cell communication, OMVs are key to biofilm formation and facilitating host-pathogen interactions, which is attributed to their proinflammatory properties (44–46). A defensive role has also been proposed, where OMVs inhibit the efficacy of antimicrobial agents that target the bacterial cell wall (24). In addition to release, the amount and composition of the OMVs were found to be altered and include proteins involved in bacterial virulence such as lipoproteins, toxins, and adhesins. Changes in OMV protein composition likely explain the altered inflammatory properties observed in the *in vivo* mouse models that support bacterial persistence in sex steroid-rich environments.

Thus, our results support a model whereby sex steroids act as extracellular chemical signals received by the surface sensing regulatory network of *P. aeruginosa* (41) (Fig. 6)

involving the *algT-muc* cluster, which is central to the control of bacterial virulence and responses to environmental stresses (47). Potentially, exposure to steroids *in vivo* over a long period may induce mutations in the *muc* operon that may reduce the fitness costs provoked by the steroid stress. This might lead to a selection of mucoid and persistent *P. aeruginosa* mutant strains that confer poorer clinical outcomes (8, 48). Further investigations are warranted to better understand the impact of sex steroid-induced OMVs on the course of *P. aeruginosa* infection, especially in the setting of a chronic inflammatory milieu.

Our findings here show clinical relevance since we observe a correlation between the membrane fluid dynamics of individual *P. aeruginosa* clinical isolates and their ability to respond to sex steroids. We show that the concentration and chemical nature of the steroid hormone shape the level of response together with the membrane properties of the exposed *P. aeruginosa* strain. This finding might explain sex differences observed across a range of lung diseases affected by chronic *Pseudomonas* infections (1, 2, 4). Females with CF have earlier colonization (8, 49–51) and mucoid conversion of *P. aeruginosa* (8, 52), which in turn, translates to poorer clinical outcomes and higher mortality (8, 52–54). However, this is not uniformly observed across individual CF females. Our work highlights that colonization with “hormone-responsive” strains may explain this phenomenon and, as such, relate to other chronic respiratory diseases complicated by *P. aeruginosa* infection. This work also furthers our understanding of the variable treatment responses observed between individual female patients affected by *P. aeruginosa*. Exploring whether a colonizing *P. aeruginosa* strain is responsive to hormonal stress may allow a more focused and tailored therapeutic approach based on sex steroids and gender, particularly in the era of antimicrobial resistance (55, 56). A further interesting aspect of our work is improved mechanistic insight into why CF females taking oral contraceptive pills experience fewer exacerbations and need for antibiotics (8, 57, 58). In these women, synthetic exogenous ethinylestradiol ([EE2] logP, 4.15) suppresses the more polar and endogenous estradiol ([E2] logP, 2.70) (59), which based on our data, would have less effect on inducing *P. aeruginosa* virulence and potentially translate to better patient outcomes.

Importantly, the observed effects on *P. aeruginosa* virulence are not specific to steroids. Colistin, an antimicrobial used in the treatment of multidrug-resistant *P. aeruginosa* infection, illustrated a comparable ability to that observed with sex steroids in modulating *P. aeruginosa* virulence. In agreement with previous studies, our data highlight the potential for colistin to exhibit deleterious effects on bacterial virulence if used at suboptimal concentrations against the targeted pathogen (60). Chemical, physiological, or therapeutic exposures including steroids and nonsteroidal or antimicrobial agents that may influence bacterial virulence and the course of infection should be considered when evaluating an optimal treatment strategy for any individual patient (61). Despite emerging evidence of potential sex bias in infection, therapeutic strategies and antimicrobial treatments remain unchanged and do not currently account for gender (1, 4, 5). Antimicrobial dosing is currently largely determined using *in vitro* and *in vivo* pharmacotherapeutic studies with environmental conditions focused on host immunity, the pathogen’s antimicrobial susceptibility, and the antimicrobial agents’ pharmacokinetic properties (62). However, patients who are treated with concomitant and sometimes combination therapies are at the same time exposed to their natural circulating hormones that, based on the data presented, confer an additional and largely unrecognized effect on pathogen virulence and evolution and antimicrobial susceptibility. Further investigations are therefore needed to better understand the impact of sex steroid hormones and other concomitant therapies (using polar molecules) that can induce a *P. aeruginosa* membrane stress response. These effects at an individual level may influence optimal antimicrobial dosing regimens and could become part of precision-medicine approaches for the treatment of infectious disease.

Gender differences are also reported with other clinically relevant Gram-negative pathogens (2, 63, 64), and existing work supports the findings presented in this study with *P. aeruginosa* (65–67). Higher bacterial survival was reported in ovariectomized

rabbits infected with *E. coli* following treatment with estradiol and progesterone (65). Similarly, female virgin mice treated with estrogen or progesterone for 3 days illustrate significant increases in *Salmonella enterica* serovar Typhimurium survival (67). Although such work was not designed to assess the direct effects of sex steroids on bacterial pathogenicity, it is of interest that an envelope stress response mechanism does exist in both *E. coli* and *Salmonella* spp. to maintain membrane integrity and cell homeostasis and facilitate chronic colonization (68, 69). These mechanisms are controlled by the extracytoplasmic sigma factor σ^{E} or σ^E , which shares 66% and 91% homology, respectively, with *P. aeruginosa* AlgU (68).

Although this study comprehensively assesses the *in vitro* and *in vivo* effects of sex steroid hormones on *P. aeruginosa* virulence, there are further aspects that warrant future investigation. We did not investigate metabolomic changes that occur under sex steroid stress. *P. aeruginosa* secretes a range of signaling molecules to communicate with its surroundings and adapt its behavior to its environment. Such molecules, termed “auto-inducers” are secreted through their membrane, transported by OMVs, and affect membrane dynamics and integrity, in turn playing a central role in the host-pathogen relationship (70, 71). Therefore, studies focusing on metabolomic alterations in the bacterial membrane, and within OMVs, should be considered in future research including the production of quorum sensing (QS) molecules. The biofilm models presented in this study may not optimally reflect the *in vivo* lung state. Air-liquid interface cell cultures may be a model for future work that more accurately mimics the *in vivo* state (72). Finally, additional *in vivo* animal models more representative of a chronic infection state, while more complex to perform, may be valuable in future work to better understand how sex steroids influence *P. aeruginosa* invasion, colonization, and adaptation in the lungs over the longer term.

Our work unlocks novel academic and translational research avenues for the microbial endocrinology field, and our findings should be carefully considered in individuals receiving concomitant high-polarity therapeutics. The influence of sex steroids on membrane stress, bacterial virulence, and antimicrobial susceptibility demonstrated here for *P. aeruginosa* should also be considered for other clinically relevant Gram-negative pathogens.

MATERIALS AND METHODS

Ethics statement. Approval for investigation of the isolates of *P. aeruginosa*, with linked pseudonymized patient metadata was given by the St. James’s/Tallaght University Hospital Ethics Committee (REC: 2017-01 list 1 [8]). Ethics approval for experiments conducted with BALB/c female mice was obtained from the University of Newcastle Animal Care and Ethics Committee (A-2012-233), and for those with C57BL/6 female mice, was obtained from the IACUC Committee of the Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences (SIAT-IACUC-181225-YYS-LL-A0551).

Bacterial strains and growth conditions. The strains of *P. aeruginosa* used in this study are listed in Table S2. The strains included *algR* and *mucA* mutant derivatives obtained from the *P. aeruginosa* PAO1 transposon mutant two-allele library (University of Washington, Seattle, WA; Manoil laboratory), which was established with NIH funds (grant P30 DK089507) (73). A *vfr* deletion mutant was generated using a previously reported knockout system (74), and *vfr* and *mucB* complemented isolates were constructed by electroporation of pNIC *vfr* or pNIC *mucB* plasmids (Bio Basic, Inc.). Mutant selection was performed on gentamicin-containing agar plates (30 μ g/ml). Bacterial strains were cultured in tryptic soy broth (TSB; Becton, Dickinson) at 37°C under aeration (200 rpm) unless stated otherwise. Medium was prepared in glass bottles previously washed with a solution of hydrochloric acid and rinsed at least twice with distilled water (dH₂O) to remove any excess detergents or ions that may affect experimental reproducibility. Stock solutions of steroid hormones (Sigma-Aldrich) were prepared in ethanol (Thermo Fisher Scientific) and stored at –20°C, and new stocks were generated every 7 days. TSDG.HCl was synthesized as described in Supplemental Methods and Fig. S7. Colistin (Sigma-Aldrich) was prepared fresh before each experiment, solubilized in sterile dH₂O, and further diluted to the desired concentrations in 100% molecular-grade ethanol (Thermo Fisher Scientific) for experimental consistency.

***P. aeruginosa* surface motility assay.** The swarming motility of *P. aeruginosa* strains was assessed using the previously described methodology with some modifications (75). Swarming medium was prepared by mixing 0.5% (wt/vol) Bacto agar (Axil Scientific, 1st Base), 0.5% (wt/vol) peptone (Becton, Dickinson), and 0.2% (wt/vol) yeast extract (Axil Scientific, 1st Base). Following agar sterilization, the medium was supplemented with 1.0% (vol/vol) glucose (Axil Scientific, 1st Base) and left to cool at 45°C in a water bath. Molten agar (20 ml) was poured into 15-cm petri dishes and dried open for 5 min in a fumigated hood. Overnight cultures (2 ml) of *P. aeruginosa* grown in the presence of ethanol (control), steroids, or colistin were centrifuged (8,000 rpm, 10 min). Pellets were spot inoculated at the center of

the agar surface using a sterile toothpick, and plates were then incubated for 16 h at 37°C. Images of swarming colonies were taken using a Gel Doc XR + system (Bio-Rad), and the extent of swarming was determined by measuring the swarming area using ImageJ software (76). At least, three biological and two technical replicates were performed to ensure the reproducibility of each experiment.

Extraction and quantification of *P. aeruginosa* virulence factors. *P. aeruginosa* strains were cultured in TSB at 37°C under aeration (200 rpm) in the presence of ethanol (control), steroids, or colistin. After measuring cell density (optical density at 600 nm [OD₆₀₀]), overnight cultures (10 ml) were centrifuged (10,000 rpm, 10 min). Supernatants were collected and filter sterilized through 0.2- μ m filters (Pall Life Sciences) and used for rhamnolipids, elastase, and alginate extraction and quantification.

Rhamnolipids. Rhamnolipids were extracted and further quantified using a previously reported methodology with modifications (77). Briefly, rhamnolipids were extracted from supernatants (8 ml) using diethyl-ether. Extraction was performed twice, and the collected organic fractions were left to evaporate overnight. The resulting white solid precipitates were dissolved in water (100 μ l) and mixed with a 0.19% (wt/vol) orcinol solution (900 μ l) freshly prepared in 50% H₂SO₄. Samples were heated at 80°C for 10 min and cooled to room temperature for 10 min before the absorbance at 421 nm was measured. The results were normalized using the OD₆₀₀ of the overnight cultures. Three biological and three technical replicates were performed to ensure experimental reproducibility.

Elastase. The elastase present in the filtered supernatants (1 ml) was measured using an elastase assay kit (EnzChek) according to the manufacturer's instructions. TSB medium was used as the negative control, while elastase provided in the kit was the positive control. Fluorescence from each reaction (expressed in relative fluorescence units [RFU]) was measured using a microplate reader (Tecan Infinite 2000) and normalized using the OD₆₀₀ of the overnight cultures. Three biological and three technical replicates were performed to ensure experimental reproducibility.

Alginates. The alginates present in the filtered supernatants were determined using the uronic acid/carbazole reaction as previously described (8). Supernatants (1 ml) were mixed with 2% cetylpyridinium chloride (500 μ l) to precipitate alginates. Alginates were then collected by centrifugation (10,000 \times g for 10 min at room temperature) and resuspended in 500 μ l of cold (-20°C) isopropanol for 1 h. Repeat centrifugation was performed (10,000 \times g at 4°C for 10 min), and the alginate pellets were resuspended in 1 M NaCl (500 μ l) for alginate quantification. A solution of purified alginate (50 μ l) was mixed with 200 μ l of sodium tetraborate/sulfuric acid reagent (25 mM in 2 M H₃BO₃ in sulfuric acid), and the mixture was heated to 100°C for 10 min. Following cooling for 15 min, 50 μ l 0.125% carbazole reagent (in 100% ethanol) was then added, and the mixture was heated to 100°C for another 10 min. Following cooling for 15 min, alginate concentrations were determined spectrophotometrically at 550 nm using a standard curve with alginic acid. Three biological and two technical replicates were performed to ensure experimental reproducibility.

Biofilm culture and imaging. Colonies of *P. aeruginosa* were suspended in fresh sterile TSB (5 ml), and inoculum (200 μ l) was distributed across 8-well Ibidi μ -slides (Martinsried, Germany). Individual samples were supplemented with either 1 μ l molecular-grade ethanol (control), steroids, or colistin at the desired concentrations. Cultures were placed at 37°C under static conditions for biofilm growth. After 16 h, biofilms were gently washed with sterile phosphate-buffered saline (PBS) and stained with Cyto-9 (LIVE/DEAD BacLight bacterial viability kit; Thermo Fisher) for 30 min. Biofilms were then washed twice with PBS before imaging using an LSM780 confocal laser scanning microscope (CLSM, Carl Zeiss, Oberkochen, Germany) at 40 \times . Representative CLSM images from each well were randomly acquired by scanning Z-stacks using ZEN (Zeiss software). All CLSM stacks were processed using Imaris software (Bitplane AG, Zurich, Switzerland) to produce projection images of the biofilms. Biovolumes were extracted from 3-dimensional (3D) biofilm images using the Imaris (Bitplane AG, Zurich, Switzerland) image analysis package, while surface roughness coefficients were analyzed from 3D confocal images of biofilm using the plug-in COMSTAT 2.1 (78) running in ImageJ (79). A total of 11 images from 3 independent experiments were acquired and processed for each condition.

Single plane illumination microscopy fluorescence correlation spectroscopy (SPIM-FCS). The protocol used was as previously described (80). Briefly, SPIM-FCS was used to quantitate the mobility of molecules diffusing in 3D in the biofilm matrix of the *P. aeruginosa* biofilm.

Flow cell setup. The biofilm setup employed is a modification of the conventional flow cell system which consists of a square fluorinated ethylene propylene (FEP) tube (2 cm long, 2.1 mm inner diameter, 2.3 mm outer diameter; Adtech Polymer Engineering Ltd., Aston Down East, Gloucestershire, UK). Flow cells were continuously supplied at a flow speed of 8 ml h⁻¹ with minimal medium [15.1 mM (NH₄)₂SO₄, 33.7 mM Na₂HPO₄, 22 mM KH₂PO₄, 0.05 mM, NaCl, 1 mM MgCl₂·6H₂O, 100 μ M CaCl₂·2H₂O, and 1 μ M FeCl₃·6H₂O] supplemented with 2 g/liter glucose and 2 g/liter casamino acid. Medium was then supplemented with either testosterone (2.5 μ M) or ethanol (control). A 5-ml liquid culture of *P. aeruginosa* PAO1 was grown overnight at 37°C (200 rpm), and bacterial cultures were diluted in minimal medium to achieve a cell density of 0.2 (OD₆₀₀). Prepared inocula were introduced into the flow cell using a 0.5-ml sterile syringe. The continuous flow was paused to allow bacterial cells to settle and attach to the flow cell. The flow resumed after 1 h, and biofilms were cultured at room temperature for 3 days.

Preparation of biofilm samples. FEP tubes containing biofilms were detached from the above-described system, and outlet ends were sealed with silicone glue (3M super silicone clear sealant). Minimal medium supplemented with 100 nM 150 kDa Dextran TRITC (TdB Consultancy, Uppsala, Sweden) was introduced into the tubing containing the biofilms and incubated for 1 h at room temperature. The inlet ends were attached to the SPIM sample capillary.

SPIM-FCS measurements. SPIM imaging was carried out using a commercially available light-sheet microscope (Zeiss Lightsheet Z.1; Carl Zeiss AG, Germany). A laser power of 1 to 3 mW was used for

illumination. LSFM10x/NA0.2 served as the illumination objective, while a WPlan-Apochromat 63x/NA1.0 was used as the detection objective. Samples were illuminated using a 561-nm laser. The fluorescence was filtered using LP565. Before the region of interest was acquired, the camera was cooled to -80°C , exposure gain was set to 300, and exposure time was set to 0.002 s. A total of 50,000 frames were collected for each sample. The ImFCS plugin (81) was used to compute the diffusion coefficient of the 150-kDa neutral molecule through *P. aeruginosa* biofilms. The data were binned 3×3 , and bleach correction was performed using a polynomial of order 4. The model used to fit the data is given below.

$$G(\tau) = \frac{1}{N} \frac{g(\tau)}{g(0)}$$

$$g(\tau) = \left\{ \frac{\sqrt{4D\tau + \omega_{xy}^2}}{a\sqrt{\pi}} \cdot \left[e^{\left(-\frac{a^2}{4D\tau + \omega_{xy}^2}\right)} - 1 \right] + \text{Erf}\left(\frac{a}{\sqrt{4D\tau + \omega_{xy}^2}}\right) \right\}^2 \times \left(1 + \frac{4D\tau}{\omega_z^2} \right)^{-\frac{1}{2}} + G_{sc}$$

where a is the pixel size (267 nm), τ is the lag time, N is the number of particles determined by fitting, D is the diffusion coefficient estimated by fitting, ω_{xy} is the e^{-2} radius in the xy direction (1.5 μm), and ω_z is the thickness of the light sheet (1.9 μm).

Bacterial transcriptomics. *P. aeruginosa* PAO1 was cultured overnight in TSB at 37°C under aeration (200 rpm) in the presence of ethanol (control) with or without testosterone (2.5 μM). Then, 1-ml cultures were treated with RNAProtect reagent (Qiagen, Germany) to maintain the integrity of the RNA. Total RNA was then extracted using an RNeasy minikit (Qiagen, Germany) with some modifications. A Turbo DNA-free kit (Thermo Fisher Scientific, Lithuania) was used to remove genomic DNA contaminants from the total RNA. DNA contamination was assessed with a Qubit double-stranded (dsDNA) high sensitivity assay (PicoGreen dye) and a Qubit 2.0 fluorometer (Invitrogen, Austria) according to the manufacturer's instructions. rRNA was depleted with a Ribo-Zero rRNA removal kit (Illumina, USA), and integrity of the total RNA was assessed with an Agilent TapeStation system (Agilent Technologies, UK). Double-stranded cDNAs were reverse-transcribed using the NEBNext RNA first- and second-strand synthesis module (NEB, USA). cDNAs were subjected to Illumina's TruSeq stranded mRNA protocol, and quantitated libraries were pooled at equimolar concentrations and sequenced on an Illumina HiSeq 2500 sequencer in rapid mode at a read length of 100-bp paired ends. Differential expression analysis of RNA-seq data was performed using the Rockhopper analysis suite with default parameters and using *P. aeruginosa* PAO1 (GenBank accession number [NC_002516](https://www.ncbi.nlm.nih.gov/nuccore/NC_002516)) and its associated annotation as a reference.

RNA isolation and Northern blot analysis. *P. aeruginosa* PAO1 was grown overnight in TSB liquid culture (180 rpm, 37°C). Bacteria were then diluted 100-fold in 10 ml TSB medium supplemented with 100 μl ethanol (negative vehicle control), testosterone (2.5 μM), estriol (25 nM), or colistin (0.4 μM). Cultures were incubated at 37°C overnight or subcultured three times (100-fold) for a total of 3 days. RNA was isolated using RNeasy Midi prep kits (Qiagen) following the manufacturer's protocol. The purity and integrity of RNA were determined by spectrophotometry. For Northern blotting, 20 μg of total RNA was separated on an agarose/formaldehyde gel as previously described (82). RNA was then transferred to Hybond N+ membranes (GE Healthcare Life Sciences) by capillary blotting. Membranes were hybridized overnight with ^{32}P γ -labeled DNA oligonucleotides (Table S2B). Northern blots were developed using a Bio-Rad Molecular Imager FX, and signals were quantified with ImageJ (1.52 h).

Outer membrane vesicles (OMV). Extraction: The protocol was described previously (83). Briefly, PAO1 was cultivated in TSB (200 rpm, 37°C) with ethanol, ethanol-dissolved testosterone (2.5 μM), estriol (25 nM), or colistin (0.4 μM). Cell-free supernatants were collected by centrifugation of the overnight cultures (10,000 rpm, 10 min, room temperature). Residual cells were removed by filtration of the supernatant through a Nalgene Rapid-Flow sterile disposable filter unit (500 ml) with PES membrane (pore size, 0.2 μm). Filtration was repeated twice to ensure that the supernatants were cell-free. Bacterial MVs were pelleted from filtered supernatants by ultracentrifugation (25,000 rpm, 1.5 to 2 h, 4°C) using a Type 45 Ti rotor. OMVs were then resuspended in 1 ml $1 \times$ phosphate buffered saline (PBS; Gibco), at pH 7.

Purification: Next, OMVs were purified using density gradient centrifugation (ranging from 35 to 60%) with Opti-Prep (Sigma Aldrich) diluted with $1 \times$ PBS. Opti-Prep bands containing OMVs were then washed twice with $1 \times$ PBS to remove the Opti-Prep solution.

Particle count and size measurement: OMV samples were analyzed using a NanoSight NS300 system to quantify the size and particle concentrations, and the Malvern Zetasizer DLS instrument was used to acquire the hydrodynamic mean vesicle size. Each sample was measured in triplicate, and data were combined for further analysis.

Proteomic analysis: Tandem MS was performed by the SciLife facility (Uppsala, Sweden). Briefly, total protein concentration was measured using Bradford protein assays (Pierce kit). For each sample, 15 μg of protein was used for digestion. Proteins were digested with trypsin according to a standard operating procedure. The collected peptides were then purified, dried, and resolved in 0.1% formic acid and further diluted 4 times. Tandem MS was performed by applying high-energy collisional dissociation. Protein identification was based on at least two matching peptides of 95% confidence per protein. Only proteins with a Sequest score above 30 were considered for analysis.

Transmission electron microscopy (TEM): Glow-discharged carbon-coated grids (Oxford Instruments, United Kingdom) were incubated for 1 min with a drop of bacterial solution or purified OMVs and negatively stained with 1% uranyl acetate in water. Images were acquired using a high-resolution JEOL ARM300 transmission electron microscope operating at 300 keV with the Gatan one-view CMOS Camera (Gatan, USA).

Fluorescence recovery after photobleaching (FRAP) assays. Cell growth and preparation: Liquid cultures (2 ml) of *P. aeruginosa* were cultivated in TSB at 37°C with aeration and shaking (200 rpm). Overnight cultures were washed three times with 1× phosphate-buffered saline (PBS) before cell exposure to a final concentration of 10 μM Nile red dye (Thermo Fisher Scientific). Samples were incubated at room temperature in the dark for 15 to 20 min. Unbound dye was removed following centrifugation (10,000 rpm, 5 min), and bacterial cells were further washed twice with 1× PBS. Then, 10 μl of cells resuspended in PBS was loaded onto a 1% agarose pad (Vivantis LE grade) prepared in 1× PBS at pH 7.4 (Gibco). Molten agar (500 μl) was sandwiched between two pieces (1 by 3 inches) of microscope glass slides (1 mm to 1.2 mm thickness), and coverslip covering pads were sealed with paraffin wax.

FRAP measurements: FRAP measurements were taken with a confocal laser scanning microscope (Carl Zeiss LSM 780) using the 561-nm line at the ×100 lens objective. A rectangular strip with a width of 0.34 μm and length extending across the width of the bacterial cell was drawn in the middle of the cell. The fluorescence within the strip was measured at low laser power (4%) and then photobleached with full laser power (100%) for 0.37 s. Fluorescence recovery was followed immediately with low laser power for ~5.7 s, the time required to reach a plateau in the fluorescence intensity.

Data analysis: Molecular dynamics were semiquantitatively characterized by fitting the FRAP curves to an exponential equation providing the half-time of recovery ($t_{1/2}$) and the mobile fraction measurements. However, our data could not be fitted in a single exponential equation. A bi-exponential equation was, instead, used to fit the curves obtained in our experiments:

$$I(t) = F_{m1}(1 - e^{-\tau_1 t}) + F_{m2}(1 - e^{-\tau_2 t})$$

where F_{m1} and F_{m2} represent the mobile fractions. The half-time of recovery was calculated from the rates τ_1 and τ_2 obtained from the fit:

$$t_{1/2} = \frac{\ln 0.5}{-\tau}$$

whereas the mobile fraction or the fraction of recovery was obtained from

$$F_m = F_{m1} + F_{m2}$$

Animal experiments. *P. aeruginosa* infection: Specific-pathogen-free, 6- to 10-week-old, female BALB/c mice were obtained from the University of Newcastle (New South Wales, Australia) central animal house. Animals were housed on a 12-h light:dark cycle in individually ventilated cages with food and water available *ad libitum*. All experimental procedures were performed under aseptic techniques and with approval from the University of Newcastle Animal Care and Ethics Committee.

P. aeruginosa (PAO1) was grown overnight (37°C, shaking) in Luria-Bertani (LB) broth supplemented with testosterone (2.5 μM; Sigma-Aldrich, Castle Hill, Australia), estriol (25 nM; Sigma-Aldrich), or ethanol (Sigma-Aldrich). Bacteria were quantified by spectrophotometry (600 nm; iMark microplate reader; Bio-Rad, Hercules, CA, USA) and regression curve analysis relating known values to optical density. Animals were intranasally infected with 10⁶ CFU of pretreated PAO1 in 30 μl of PBS under light isoflurane anesthesia. Animals were then euthanized by lethal intraperitoneal injection of sodium pentobarbitone at 0.5 and 6 h postinfection. BALF was then collected from the airways by lavaging the lungs twice with 0.5 ml of PBS. Lavaged lung tissue was then collected into 1 ml of PBS and homogenized on ice. BALF and homogenized lung tissue were plated onto LB agar plates (20 μl, 5 × 1:10 serial dilutions) and incubated overnight (37°C, 24 h), and the CFU were counted to determine bacterial titers in tissues. Results are presented as combined BALF and lung bacterial titers and those from individual tissues (84–86). Cells were collected from the BALF (295 × g, 4°C, 7 min), with red blood cells lysed (Tris-buffered NH₄Cl, 500 μl, 5 min). The remaining leukocytes were collected (295 × g, 4°C, 7 min) and counted using a hemocytometer. The leukocytes were cytospun (300 rpm, 10 min; Thermo Fisher Scientific, Scoreby, Australia) onto microscope slides, air-fixed, and then stained with May-Grunwald-Giemsa. Differential counts of inflammatory cells were performed under light microscopy (×40 magnification) and determined by size and morphology (84).

OMV *in vivo* challenge: For OMV experiments, all experimental procedures were performed under aseptic techniques and with approval from the IACUC committee of the Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences. C57BL/6 mice (female, 6 to 8 weeks old) underwent intratracheal installation of OMV extracts in 70 μl sterile saline using a standardized concentration of a total of 10 μg protein. Twenty-four hours after OMV administration, BALF was harvested by flushing 1 ml of sterile saline into the lungs through a catheter inserted into the mouse trachea. Injected saline was retracted with a 3-ml syringe, centrifuged at 3,500 rpm, and resuspended in red blood cell lysis buffer (Beyotime; C3702). Supernatant from the saline centrifugation was collected for Luminex multicytokine analysis using a Bioplex 23-cytokine mouse panel (Bio-Rad; M60009RDPD). The lung tissues were then dissected and fixed in 4% paraformaldehyde before serial dehydration in ethanol, xylene, and paraffin. Paraffin blocks were sectioned at 5 μm, stained for hematoxylin and eosin, and processed for histology.

Statistical analysis. Unless stated otherwise in the experimental procedures, statistical significance was determined by one-way analysis of variance (ANOVA) corrected using Dunnett's test for multiple comparisons. Differences were considered statistically significant when the *P* values were <0.05. Analysis was performed using GraphPad Prism 7.0.

Data availability. The MS proteomics data related to potential steroid-binding proteins were deposited in the ProteomeXchange Consortium via the PRIDE (87) partner repository with the data set identifiers PXD018897 and 10.6019/PXD018897. Reviewer account details are as follows: username, reviewer96473@ebi.ac.uk; password, kQhOxvYM. The MS proteomics data related to OMVs were depos-

ited into the ProteomeXchange Consortium via the PRIDE (87) partner repository with the data set identifier PXD019470. Reviewer account details are as follows: username, reviewer94411@ebi.ac.uk; password, kl78AUPh. The transcriptomics data discussed above have been deposited in NCBI's Gene Expression Omnibus (88) and are accessible through the GEO Series accession number [GSE148955](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148955). These 3 data sets were used to generate transcriptomic data (Fig. 2A) and proteomic data for steroid protein binders (Table S1A) and the OMV protein profile (Table S1B).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.03 MB.

FIG S1, TIF file, 2.3 MB.

FIG S2, TIF file, 1.8 MB.

FIG S3, TIF file, 2.9 MB.

FIG S4, TIF file, 2.9 MB.

FIG S5, TIF file, 2.2 MB.

FIG S6, TIF file, 1.7 MB.

FIG S7, TIF file, 2.3 MB.

TABLE S1, DOCX file, 0.03 MB.

TABLE S2, DOCX file, 0.02 MB.

ACKNOWLEDGMENTS

This research was supported by the Singapore Ministry of Health's National Medical Research Council under a clinician-scientist individual research grant (MOH-000141) awarded to S.H.C., the Lee Kong Chian School of Medicine (LKCMedicine), Nanyang Technological University, Singapore Start-Up grant to S.H.C., a grant from LKCMedicine to B.H.N. and S.N., the Swedish Research Council, the Knut and Alice Wallenberg Foundation and the Swedish Foundation for Strategic Research (SSF) to B.H.N. and S.N., and a start-up grant from LKCMedicine to B.C. (M4082195).

We acknowledge the Facilities for Analysis, Characterization, Testing, and Simulations (FACTS) at Nanyang Technological University for access to TEM equipment.

P.M.H. is supported by a fellowship and grants from the National Health and Medical Research Council (NHMRC) of Australia (1175134).

We thank Peter Mellroth (Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden) for helpful discussions and Martial Duchamp (School of Materials Science and Engineering [MSE], Nanyang Technological University, Singapore) for his valuable assistance with the TEM experiment.

The University of Washington *P. aeruginosa* transposon mutant library is supported by NIH P30 DK089507.

C.V. planned and conceived all the *in vitro* and *in vivo* experiments and performed the *in vitro* experiments and data analysis. V.F.L.Y. performed all the *in vitro* experiments and data analysis. C.V. and V.F.L.Y. drafted the first draft of the manuscript. M.-S.A. significantly contributed to the design of all *in vitro* and *in vivo* experiments and the interpretation of all the results, and performed the OMV proteomic analysis and TEM experiments. Z.J.S. and L.L. constructed the *vfr* mutant strain and plasmid for complementation and performed the RNA sequencing. A.C.B., M.K.A., J.C.H., and P.M.H. performed the *P. aeruginosa in vivo* infection experiments. J.Q., S.Y., G.X., and L.L. performed the *in vivo* experiments with OMVs. T.K.J. contributed to the *in vitro* virulence assays. A.M.N. analyzed the OMV samples for purity and size. J.S. and T.W. performed, analyzed, and interpreted the FCS experiments. Y.H.F. performed and analyzed the FRAP experiments. F.R. and E.L. designed, performed, and analyzed the Northern blot experiments. M.M.A. analyzed the RNAseq data sets. D.R. contributed to the acquisition and analysis of the biofilm confocal images. J.-A.R. designed and performed the synthesis and analysis of the compound TSDG.HCl. T.R.R. provided clinical isolates of *P. aeruginosa* and contributed to collecting and obtaining data related to the strains. M.T. provided wild-type PAO1 and its *mucB* knockout derivative and contributed to collecting and obtaining data related to these strains. D.L. supported and advised all ligand-protein interaction experiments. B.C. prepared, acquired, and analyzed samples for

TEM. S.N. and B.H.N. contributed to the interpretation of the data, the relation to other literature, and the revision of the manuscript. S.H.C. managed the entire project, procured funding, and was involved in the conception, design, and execution of all experiments. He wrote the final manuscript with contributions from all coauthors.

T.R.R. has been advisory board chair and member for, and recipient of conference travel bursary from, A Menarini Pharmaceuticals, Ireland, as well as receiving lecture honoraria from Pfizer Healthcare Ireland and Gilead Sciences, Inc. All other authors have no conflicts of interest.

Note Added after Publication

In the initial publication (on 29 September 2020), there were minor errors in the byline and affiliations. The article was corrected online on 27 October 2020.

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Erratum for Vidailiac et al. "Sex Steroids Induce Membrane Stress Responses and Virulence Properties in *Pseudomonas aeruginosa*"

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Volume 11, no. 5, e01774-20, 2020, <https://doi.org/10.1128/mBio.01774-20>. Professor Birgitta Henriques-Normark's last name was missing a hyphen between Henriques and Normark in the original article. The byline should appear as shown above. Also, the affiliations and affiliation letters are correctly represented herein.

Citation Vidailiac C, Yong VFL, Aschtgen M-S, Qu J, Yang S, Xu G, Seng ZJ, Brown AC, Ali MK, Jaggi TK, Sankaran J, Foo YH, Righetti F, Nedumaran AM, Mac Aogáin M, Roizman D, Richard J-A, Rogers TR, Toyofuku M, Luo D, Loh E, Wohland T, Czarny B, Horvat JC, Hansbro PM, Yang L, Li L, Normark S, Henriques-Normark B, Chotirmall SH. 2020. Erratum for Vidailiac et al. "Sex steroids induce membrane stress responses and virulence properties in *Pseudomonas aeruginosa*." mBio 11:e02809-20. <https://doi.org/10.1128/mBio.02809-20>.

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Published 3 November 2020