Circadian rhythms in the critical human pathogen Acinetobacter baumannii

María Alejandra Mussi

mussi@cefobi-conicet.gov.ar

Centro de Estudios Fotosintéticos y Bioquímicos - CONICET https://orcid.org/0000-0002-4168-3624

Valentín Permingeat

Centro de Estudios Fotosintéticos y Bioquímicos - CONICET

Bárbara Perez Mora

Centro de Estudios Fotosintéticos y Bioquímicos - CONICET

María Migliori

https://orcid.org/0009-0004-3588-8430

Natalia Arana

Julia Fernández

Facultad de Ciencias Bioquímicas y Farmacéuticas https://orcid.org/0000-0001-6600-1442

María Belén Allasia

Melisa Lamberti

Univerisad Nacional de Quilmes

Gisela Di Venanzio

Mario Feldman

Washington University in St. Louis

Diego Golombek

Universidad de San Andrés

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Abstract

Acinetobacter baumannii is recognized as the paradigm of multidrug resistant superbug, topping the WHO priority list of critical human pathogens. Interestingly, it senses and responds to blue light, which modulates global aspects of its physiology including the pathogenicity. We hypothesized that light could serve as a signal to synchronize the bacterial physiology to the host's behavior, or to the environment. At environmental temperatures, light regulation is mainly governed by the BLUF-type photoreceptor BlsA. In this work, we identified the existence of daily rhythms in *blsA* expression displaying a robust response to light, as well as endogenous circadian rhythms in *A. baumannii*. In fact, we show that *blsA* gene expression can be synchronized to 24-hour blue light-dark cycles, which immediately resynchronizes after a phase shift due to a longer night. Upon release to constant darkness, bacterial populations present free-running oscillations with a period close to 24 hours. Furthermore, our data indicate that BlsA is involved in synchronization to the zeitgeber during light-dark cycles. Importantly, β-lactamase activity varied along the day in cultures under light-dark period, establishing a new paradigm. Our work contributes to the developing field of circadian clocks in bacterial human pathogens, which could impact the microorganisms' lifestyle and pathogenicity.

Introduction

Circadian rhythms, observed across all domains of life, enable organisms to anticipate and prepare for daily changes in environmental conditions resulting from the periodic movement of the Earth. Among bacteria, circadian rhythms have only been extensively studied in cyanobacteria, which are photosynthetic microorganisms. In fact, genes responsible for the core clock of cyanobacteria have been identified, including *kaiA*, *kaiB*, and *kaiC*, along with other crucial components like the response regulator RpaA and two regulatory histidine kinases, CikA and SasA [1]. Recently, it has been shown that non-photosynthetic soil microorganisms, such as *Bacillus subtilis*, exhibit a light-dependent circadian system that behaves in a complex fashion, similar to the circadian systems of multicellular eukaryotes [2,3]. Moreover, it was shown that a clinical isolate of the gut bacterium *Klebsiella aerogenes*, exhibited melatonin and temperature-dependent rhythms in the expression of the motility gene *motA* [4,5]. Although the relevance of these circadian rhythms in pathogenesis has not been explored, these findings suggest that the circadian clock of *K. aerogenes* may entrain to host cues *in vivo*.

Although light-dependent circadian rhythms have not been reported in bacterial pathogens, it is well established that important pathogens such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Brucella abortus*, and *Staphylococcus aureus* sense and respond to light, showing a global photic modulation of bacterial physiology and, most interestingly, of determinants of pathogenicity [6–11]. A large body of evidence demonstrates that light signal transduction and physiological responses are mainly governed by the blue light using FAD (BLUF)-type photoreceptor BlsA at environmental temperatures in *A. baumannii* [6,7,12]. In fact, blue light modulates iron uptake, tolerance and susceptibility to antibiotics, desiccation tolerance, competition with other microorganisms, antioxidant enzyme levels, metabolism,

biofilm formation, and quorum sensing [6,7,13–18]. BlsA is a global regulator able to bind and antagonize the functioning of different transcriptional regulators such as Fur, the iron metabolism repressor; and AcoN, the acetoin catabolism repressor, in a light-dependent manner [15,16]. We have also shown that *A. baumannii* responds to light modulating virulence in a human keratinocyte epithelial infection model [7] as well as quorum sensing [17] at 37ºC, through a BlsA-independent mechanism [7,17]. Moreover, the two-component system BfmRS has been shown to transduce light signaling as it is involved in photoregulation of motility and desiccation tolerance in *A. baumannii* strain V15, while evidence strongly indicates that BfmR phosphorylation levels are also modulated by light [18]. Finally, BfmRS antagonizes *blsA* transcription [18], and has been shown to physically interact with BlsA [19].

In this work, we studied the existence of circadian rhythms in the critical pathogen *A. baumannii*, which presents an alarming propensity to develop multi-drug resistance [17–20] leading to serious outbreaks in the hospital setting [20,21]. Infections associated with multi-drug resistant (MDR) *A. baumannii* are linked to higher rates of morbidity and mortality [18, 20]. Moreover, there are increasing reports of community-acquired *A. baumannii* infections across the globe, suggesting the existence of extra-hospital reservoirs. Accordingly, carbapenem-resistant *Acinetobacter* recently topped the WHO priority list of bacteria that require research and development of novel therapeutic strategies [22].

Our results show rhythmic oscillations in *blsA* expression presenting a robust response to blue light configuring daily rhythms, as well as endogenous circadian rhythms under constant conditions. In fact, we show that *blsA* gene expression can be synchronized to 24-hour blue light-dark cycles, which resynchronize immediately after a phase shift due to a longer night. Upon release to constant darkness and temperature conditions, bacterial populations present free running (FR) oscillations with a period close to 24 hours showing a phase shift from light-dark conditions, possibly indicative of circadian masking. Our data also indicate that the photoreceptor BlsA is involved in synchronization to the zeitgeber during light-dark cycles but does not influence the endogenous rhythm. Importantly, we detect differences in β-lactamase activity along the day in the light-dark period, establishing a new paradigm.

The existence of such rhythms could impact virulence, antibiotic susceptibility or persistence, opening new paradigms for treatment of infections produced by these pathogens.

Results

Rhythmic oscillations in blsA expression.

We have shown that blue light exerts a global modulation of *A. baumannii*'s physiology at moderate temperatures such as 23 ºC through the *blsA* photoreceptor [6,7,12,14–18,23]. To study the existence of rhythms in *blsA* expression in light-entrained and dark-released cultures, *A. baumannii* V15 strain cells were inoculated in LB broth, placed in 24 well-plates and incubated under a 12 h blue light (bL)- 12 h dark (D) 12L:12D photoperiod for 5 days and then released to constant darkness at 23ºC. Samples corresponding to individual wells were recovered at 4 hour-intervals for 4 days corresponding to the 4th

and 5th days of bLD entrainment as well as the 1st and 2nd days in constant darkness (DD), and *blsA* RNA levels were quantified by retrotranscription (RT) followed by quantitative PCR (qPCR), RT-qPCR, using *blsA* specific primers [14].

Our data show oscillations in *blsA* expression along different times of the day both in entrained as well as dark-released cultures (Fig. 1). A JTK_Cycle analysis implemented in the R package MetaCycle further indicated rhythmic components in *blsA* oscillations [24] (Fig. 1). Overall, our results show the existence of rhythmic oscillations in expression of the *blsA* gene determined by RT-qPCR.

Daily and free-running rhythms of blsA promoter activity in A. baumannii.

To validate the above results and characterize the rhythms more accurately, we next evaluated *blsA* promoter activity using a luciferase reporter transcriptional fusion. For this, *A. baumannii* V15 pLPV1Z-*pblsA::luc* clone 1 cultures were grown stagnantly in LB broth in 12L:12D cycles for 4 days and then released to constant darkness for 2.5 more days, at 23ºC. This strain expresses a luciferase reporter under the control of the *blsA* promoter from the pLPV1Z plasmid [25]. Results shown in Fig. 2A represent the total of wells (light blue shadow) and average (blue line) from one representative experiment of a total of 3 biological replicates.

We first analyzed whether blue light could act as a zeitgeber in *A. baumannii*. The *pblsA::luc* reporter activity showed that *blsA* expression responds to LD cycles establishing a robust diurnal rhythm with a clear phase relationship with the zeitgeber, in which expression increases during the dark phase and decreases during the light phase (Fig. 2A). The higher or lower maximal values of gene expression tend to anticipate zeitgeber transitions, suggesting that the bacteria are not only passively responding to external stimuli (Fig. 2A). Interestingly, a free-running (FR) rhythm in *blsA* promoter activity was maintained when the cultures were released to DD (Fig. 2A), showing a calculated period of 26 ± 2.4 hours (Fig. 2B), indicating the existence of a genuine circadian component.

Under LD conditions, the bioluminescence peaks occurred at zeitgeber time (ZT) 22.9 ± 0.8 h, which shifted to circadian time (CT) 14.6 ± 1.5 h under DD (Fig. 2C). The fact that the circadian phase was not maintained when cultures were transferred to DD (Fig. 2C), suggests that canonical light-entrainment is not occurring but rather a zeitgeber masking effect is taking place. In other words, the blue light zeitgeber is able to synchronize *blsA* expression rhythms with a robust and clear phase relationship, which is lost under constant conditions although the bacterial populations still remain rhythmic, indicating the presence of an endogenous circadian oscillator.

Figure 2D shows luminescence data retrieved from individual wells representative of the three independent experiments performed. Supplementary Fig. 1 shows similar experiments as above but performed using strains *A. baumannii* V15 pLPV1Z-*pblsA::luc* clones 2–4, recovered from independent transformation events. The data for these clones is consistent with that shown for clone 1.

A. baumannii instantaneously resynchronizes to changes in the blue light zeitgeber.

To further characterize the rhythm response to blue light in *A. baumannii*, we lengthened the third LD night by 6 hours so that the bacteria faced 18 hours of darkness, followed by reestablishment of the 12bL:12D photoperiod for additional 3 days and a final release to darkness for 2 days. Figure 3 shows a robust adaptation of the *A. baumannii*'s daily rhythm to the zeitgeber, presenting an instantaneous resynchronization to the zeitgeber's new phase. It is interesting to note that the amplitude of the peaks remained conserved respect to the previous LD days before night lengthening, indicating that *blsA* expression is not affected by culture aging or nutrient deprivation. From this, it follows that the reduction in amplitude observed when cultures are released to DD in Fig. 2A or D is a characteristic of the endogenous rhythms and not an artifact resulting from those conditions. Finally, when cultures were released to DD after resynchronization, endogenous circadian rhythms were observed, while the phase was not conserved with respect to the new LD phase, indicating masking and consistency with previous results shown in Fig. 2 and Supplementary Fig. 1.

Overall, there is a strong response to the blue light zeitgeber in *blsA* expression, where masking appears as a predominant component leading to instantaneous resynchronization, again showing endogenous circadian rhythms under constant illumination conditions.

Rhythms are maintained under constant dark conditions in A. baumannii.

To further explore endogenous circadian rhythms in *A. baumannii*, we next decided to study the *blsA* promoter activity under constant dark conditions measuring emitted bioluminescence at discrete time points for at least 3 continuous days spanning a total of 6 days in at least 4 independent experiments. Representative experiments are shown in Fig. 4, in which the presence of rhythmic oscillations is observed in *blsA* expression with a calculated period of 23.45 h. Thus, endogenous circadian rhythms are observed under constant dark conditions. Supplementary Fig. 2 shows results for the other independent experiments.

BlsA is involved in synchronization to blue light in A. baumannii.

We further evaluated the behavior of another *A. baumannii* strain, ATCC 17978, under similar conditions as those described above. Our results show that 17978 harboring plasmid pLPV1Z-*pblsA::luc* presented a robust response to the light zeitgeber during the LD photoperiod similarly to the V15 strain, with a nocturnal increase in expression (Fig. 5A). Moreover, the presence of an endogenous rhythm was observed in DD, which exhibited characteristics of canonical light entrainment (64% of synchronized and rhythmic wells were also entrained; Fig. 5A). On the other hand, the *ΔblsA* mutant strain induced a significant decrease in circadian amplitude under LD conditions, while the endogenous rhythm was maintained in DD (Fig. 5A-B). Most interestingly, synchronization to the light zeitgeber as well as entrainment were both compromised in *ΔblsA* mutant populations (approximately 30% of wells; Chi-square test, $p < 0.005$), consistent with synchronization being required for entrainment. The acrophase dispersion to rhythmic populations was higher in the LD for 17978 *ΔblsA* strain (Watson-Wheeler test for homogeneity of angles, p = 0.0312, *$p < 0,05$) (Fig. 5C). We did not observe any significant differences in the circadian period in *ΔblsA* mutant (24.5 ± 1.1 h, n = 12 mutant rhythmic, n total = 36, 33% rhythmic vs.

24.8 ± 1.4 h, n = 14 control rhythmic, n total = 23, 61% rhythmic) (Fig. 5D). Thus, the overall results indicate that BlsA is involved in synchronization to the *zeitgeber* and entrainment, but does not affect the endogenous rhythm component.

β-lactamase activity fluctuates along the day.

A. baumannii is the paradigm of bacterial multidrug resistance, as circulating strains are invariably resistant to last generation antibiotics including the β-lactams carbapenems, which seriously complicate therapeutics. As we previously showed the existence of daily rhythms in this microorganism, we decided to evaluate next whether β-lactamase activity also oscillates along the day. For this purpose, we used the *A. baumannii* multidrug resistant strain Ab825, since both V15 and ATCC 17978 are sensitive to multiple drugs. Ab825 cells were grown under 12L:12D photocycle for 4 days, and β-lactamase activity levels were determined at two different times along the day of the 3rd and 4th days (LD3 and LD4): 7 am and 7 pm, which reflect the end of the dark and light phases, respectively. It should be noted that we performed β-lactamase activity determination to acquire an instantaneous measurement at different hours of the day, in a growth-independent manner. Our results show oscillations in β-lactamase activity levels along the day both in L3 and L4 (mean ratio of the ratio/OD different for at least one measurement time, p = 0.0045), which were higher at the end of the light phase respect to the end of the dark one (post-hoc multiple comparisons significant at 10%, Fig. 6). These results indicate that oscillations are observed not only at the gene expression level but also in cellular processes as important in *A. baumannii* lifestyle as is antibiotic-inactivating activity. These findings establish a new paradigm.

Discussion

It has been recently ascertained that many non-phototrophic pathogens are capable of sensing and responding to light via specialized molecular systems such as photoreceptors. In fact, we have extensively shown that the critical human pathogen *A. baumannii* presents a global response to light, including modulation of pathogenicity and virulence. This prompted us to determine whether this response to light is actually part of a more specialized mechanism such as a light-dependent circadian pacemaker. In this work, we provide evidence indicating that *blsA* expression shows a robust response to LD cycles leading to daily rhythms, while endogenous free-running rhythms were observed under constant darkness.

Daily rhythms are very important as they show variations in bacterial physiology due to changing environmental conditions throughout the day, while the existence of endogenous rhythms indicate that this pathogen has evolved mechanisms to anticipate periodic variations in these conditions.

Interestingly, our data show no phase conservation between LD and DD in V15 strain, suggesting that canonical light entrainment of *blsA* expression is not taking place in this case, but rather a masking mechanism induced by the zeitgeber. Masking is an important evolutionary mechanism that allows an organism to respond to changes in exogenous stimuli (e.g., light-dark cycle, social cues, temperature,

food, drugs), thereby enabling the organism to act immediately and appropriately [26]. In addition, masking can complement and integrate with entrainment if the cues align with arousal and the circadian system [26].

The circadian response to light depends on the timing of light exposure, as well as its intensity, duration, wavelength, and prior light exposure history. For example, the threshold for human entrainment requires relatively bright light for a long duration; on the contrary, the circadian system of mice is exquisitely sensitive to light [27]: a 12L:12D photocycle using light intensities as low as 0.01–0.1 lux will photoentrain murine rhythms. While it is possible that the minimum light timing information necessary for entrainment has not been reached in our experiments, this possibility would be unlikely in our setup given the robust response observed in the LD cycle. On the other side, short-wavelength ('blue') light is significantly more effective for photoentrainment compared to longer wavelengths of light [27]. In fact, blue light sensing is an integral part of circadian rhythms in all the experimental models examined so far. Moreover, it should be noted that we are using very low light intensity (8 μE m^{-2} s^{-1}), more than 4 times lower than that reported in photoentrained circadian rhythms evidenced in *B. subtilis* [2]. Thus, *a priori*, we would not expect the blue light intensity used in our setup to be too high to lead to masking effects prevailing over entrainment. Also interesting is that in DD the whole bacterial population behaves similarly, i.e. *blsA* expression increases and decreases following the same pattern in different wells, indicating that they are synchronized to some external clue, which our experiments suggest is environmental light. Indeed, as non-photosynthetic bacteria constitute poorly characterized organisms in terms of circadian rhythmicity, we can conclude that synchronization by light is a conserved mechanism, rather than phase conservation between LD and DD. In fact, bacteria introduce a new dimension in the understanding of circadian rhythms. It would thus be not surprising that non-canonical features are discovered regarding circadian rhythms in these microorganisms. It is also likely that different strains present differential characteristics regarding synchronization and entrainment by light, as well as endogenous circadian components; most probably because of differential lifestyle, antibiotic susceptibility profile, pathogenicity, etc. Such is the case of strain ATCC 17978, which, in contrast to V15 strain, shows not only synchronization but also entrainment of the endogenous rhythm.

Moreover, it is possible that the entry route is also part of the clock input pathway; in other words, BlsA could serve both as the photoreceptor sensing blue light and entraining the clock. In fact, our data indicate that the photoreceptor BlsA is involved in synchronization to the zeitgeber during light-dark cycles as well as in entrainment. In this work, we characterized *blsA* as it is the photoreceptor governing photoregulation at environmental temperatures in *A. baumannii*. Further work studying other genes and phenotypic responses will lead to gain full understanding into rhythms in this critical pathogen.

A. baumannii's rhythm was detected in conditions compatible with biofilm formation, as occurred with *B. subtilis* [2], which is considered a bacterial social behavior. In this context, another interesting aspect to further explore is the communication among these "independent individuals" that constitute the bacterial population. Several questions arise, such as whether the bacteria interact through quorum sensing, or

how is the circadian mechanism transmitted to new generations in dividing short-lived bacteria, considering that the circadian clock is thought to be dependent on state variables, i.e., substances that reflect time, whose concentrations might be disrupted by the cell division process. In this sense, in cyanobacteria a memory effect has been identified, which spans over the cell cycle interval (i.e., the physiological state of a bacterium is sustained for several cell divisions) [25]. In fact, acyl-homoserin lactones, the molecules signaling *quorum* sensing in Gram-negative bacteria, shares with melatonin the presence of specific motifs with associated functional groups. Since melatonin has been shown to couple circadian rhythms (e.g., Pilorz *et al.*, 2020 [28]), we suggest the intriguing hypothesis that *quorum* sensing might serve a similar function in bacteria.

A. baumannii is a recent human pathogen, i.e., it is known as an old friend but a new human enemy whose pathogenic character originated from selection due to extensive antibiotic use during the last decades [29]. Therefore, the possibility that it evolved a circadian rhythm to synchronize to the human host is not very straightforward. Yet, *A. baumannii* is a dual microorganism capable of environmental as well as pathogenic lifestyles. Interestingly, and despite *A. baumannii* is mainly described as a non-internalizing pathogen, the ability to invade the host's cells is increasingly recognized in modern strains [30].

This work contributes to establishing that bacterial pathogens are subjected to circadian regulation, which defines a new paradigm, and is the first reporting rhythmicity in *Acinetobacter*, which opens a new full area of research that will likely lead to reinterpretation of previous data on the pathogen's behavior. We expect this work will inspire future efforts to investigate whether bacterial pathogens can synchronize their behavior to the host's circadian rhythm and its immune response, to optimize infection or its persistence in the environment. This would not be surprising given that it has been recently shown that internal timekeeping mechanisms in the malaria parasite synchronize with the host's circadian rhythm. An example of this is the synchronization of the rupture of red blood cells with the completion of the parasite's asexual cell cycle [31]. Most interestingly, the significance is that bacterial circadian rhythms could potentially impact bacterial persistence in the environment, virulence or antibiotic susceptibility, as we have shown that the activity of β-lactamases fluctuate along the day. Changes on bacterial antibiotic susceptibility, infection outcomes or persistence in the environment influenced by the time of the day could introduce modification of treatment schedules to optimize medical interventions and prevention of critical infections, offering new opportunities for the development of targeted therapeutic strategies to combat infectious diseases and constitute a change in paradigm.

Our work contributes to the developing field of circadian clocks in bacterial human pathogens, indicating the existence of daily as well as endogenous circadian rhythms in a critical pathogen, which could impact the microorganisms' lifestyle and its pathogenicity.

Methods

Strain and constructions. The promoter region of the *blsA* gene was amplified using primers *blsA_Eco*RI_FW (5'- GAATTCagtattacaaattgaacgtgt – 3') and *blsA_Bam*HI_REV (5'- GGATCCaagacttccgtgaaatataaa – 3'). High fidelity polymerase chain reaction products were digested with *Eco*RI and *Bam*HI enzymes (Promega) and cloned into the corresponding sites of pLPV1Z harboring the promoterless *luxABCDE* genes [25]. The correct construction was verified by sequencing the cloned fragment and pLPV1Z-P*blsA*-luc was subsequently incorporated into *A. baumannii* V15 strain by transformation.

Light settings. Samples were exposed to blue light emitted by nine-LED (light-emitting diode) arrays with an intensity of 6 to 10 µmol photons/m^2/s and peak emission centered at 462 nm [6]. Light intensity was measured using a radiometer/photometer (Flame-T, OceanOptics). Temperature was set at 23ºC and fluctuations in the incubator were less than 0.5°C.

Zeitgeber (i.e., “time giver” or entraining agent) time 0 or ZT0 (9:00 am) indicates the time at which lights were turned on. Circadian Time (CT) refers to a specific time in the free running conditions (constant darkness, DD, and constant temperature of 23ºC). Photo and thermal conditions were controlled with an I-291PF incubator (INGELAB, Argentina) and temperature was monitored using DS1921H-F5 iButton Thermochrons (Maxim Integrated, USA).

Luminescence assays. For all assays, *A. baumannii* V15 or ATCC 17978 cells harboring plasmid pLPV1Z-P*blsA*-luc were cultured in white 96-well plates (Greiner) under stagnant conditions in LB broth (250 µl for well) at 23°C from an initial OD_{660} of 0,05. Plates were sealed with a transparent optical film (ThermalSeal RT2RRTM, EXCEL Scientific) to avoid evaporation and contamination, and the seal over each well was perforated twice to avoid condensation and allow oxygen exchange. Cultures were exposed to 12 h blue light (bL) and 12 h dark (D) photoperiod (12L:12D) for 4 days and then released to constant darkness, at a constant temperature of 23°C. We measured bioluminescence using a Berthold Centro LB 960 microplate luminometer (Berthold Technologies) stationed inside an incubator (INGELAB) to allow tight control of the blue light and temperature in each experiment. Microwin 2000 software version 4.43 (Mikrotek-441 Laborsysteme) was programmed to leave the plate outside the luminometer after each recording to expose *A. baumannii* V15 to the environmental cues. The luminescence of each well was integrated for 10 s every 30 min. Temperature fluctuations in the incubator due to lights being on or off were less than 0.5°C.

For phase-shift assays, *A. baumannii* V15 was entrained for 3 days under a bLD cycle and then were subjected to a phase shift caused by a 6-h night extension. After 3 more days, *A. baumannii* V15 was released into free running (FR) conditions for 2 days.

Data acquisition and analysis. Luminescence was sampled at 30 min intervals. Background noise was extracted from the raw data obtained from the luminometer. In all cases, the first 24 to 36 hours of recording were removed due to accumulation of the luciferase enzyme. All raw data was analyzed using the CircaLuc v0.7 program (https://ispiousas.shinyapps.io/circaluc/). The raw data was detrended,

smoothed and normalized to the initial maximum value of each sample and plotted using the same program. The data is shown as mean ± SD of luminescence. Subsequently, the circadian period was calculated from the data using the Lomb-Scargle (LS) periodogram within the lomb R package (DOI: 10.1076/brhm.30.2.149.1424). In the case of FR rhythms, any signal resulting from the analysis with a period range between 18 h and 33 h, and an R2 adjustment ≥ 0.5 was considered “Circadian”. Final figures were generated using Biorender (https://app.biorender.com/). Background signals lower than 10 folds of magnitude resulting from the bacteria transformed with the empty plasmid or LB broth alone were discarded from the analysis.

qRT-PCR bacteria. *A. baumannii* V15 cells were cultured in 24-well microplates under stagnant conditions in LB broth at 23 ºC from an initial OD_{660} of 0,05. The bacteria were incubated for 5 days under 12L/12D photoperiod and then released to constant darkness. 2 ml samples were retrieved every 4 hours from the 4th day of LD entrainment until and including the 2nd day released to darkness. The samples were centrifuged and the pellets were saved at -80ºC until further use. RNA was extracted following procedures described in Muller *et al.*, 2017 [14].

qRT-PCR data analyses. *blsA* expression data from the last day under LD entrainment, the first day released to darkness and the 2 following points was analyzed using JTK_CYCLE [24] and Lomb-Scargle [32] methods, implemented in the R package MetaCycle [33]. *p*-values from both procedures were integrated using Fisher’s method, while estimates of period and phase parameters were combined using the arithmetic and circular mean, respectively.

RNA extraction. RNA was extracted following procedures described in Mûller *et al.*, 2017 [14].

Instantaneous β-lactamase activity determination along the day in LD cultures.

A. baumannii Ab825 cells were grown overnight in LB at 37 ºC in the dark, and then inoculated in fresh new LB media at a DO_{600} = 0.05. The bacteria were then grown under 12L:12D photoperiod for 4 days at 23ºC. At 7 am and 7 pm of the 3rd and 4th days (LD3 and LD4), samples were retrieved and processed using a nitrocephin-based colorimetric method for β-lactamase activity detection following the manufacturer recommendations (Amplite Colorimetric Beta-Lactamase Activity, AAT Bioquest). The reactions were incubated at room temperature, with the plate protected from light, and after 60 minutes absorbances at 490, 380, and 600 nm were determined using in a microplate reader (Bio Tek Instruments EPOCH2T). A 490/380 ratio was calculated using these values and normalized to the OD_{600} corresponding to the time point for each sample. Four replicates of the experiment were carried out, one of them only measured on the third day.

Statistical analysis. For the analysis of antibiotic susceptibility, the effect of measurement time on the mean of ratio/OD was analyzed using a mixed effects model with replicates included as a random effect. Residuals of the fitted model confirmed that necessary assumptions were met. Post-hoc pairwise mean comparisons were conducted using the Kenward-Roger correction.

Declarations

Competing interests

The authors declare no competing interests.

Author Contributions

Conceptualization: V.P., B.E.P.M., M.L.M., D.A.G. and M.A.M. Formal analysis: V.P., B.E.P.M., M.L.M., N.A., M.L.L., J.I.F., M.B.A., D.A.G. and M.A.M. Funding acquisition: M.A.M and D.A.G. Investigation: V.P., B.E.P.M., M.L.M., M.L.L., J.I.F., M.B.A., G.D.V., M.F., D.A.G. and M.A.M. Methodology: V.P., B.E.P.M., M.L.M., N.A., D.A.G. and M.A.M. Project administration: M.A.M. Visualization: V.P., M.L.M., J.I.F., M.B.A. and M.A.M. (Writing—original draft: M.A.M and D.A.G. Writing—review & editing: V.P., B.E.P.M., M.L.M., J.I.F., M.B.A., G.D.V., M.F., D.A.G. and M.A.M.

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Figures

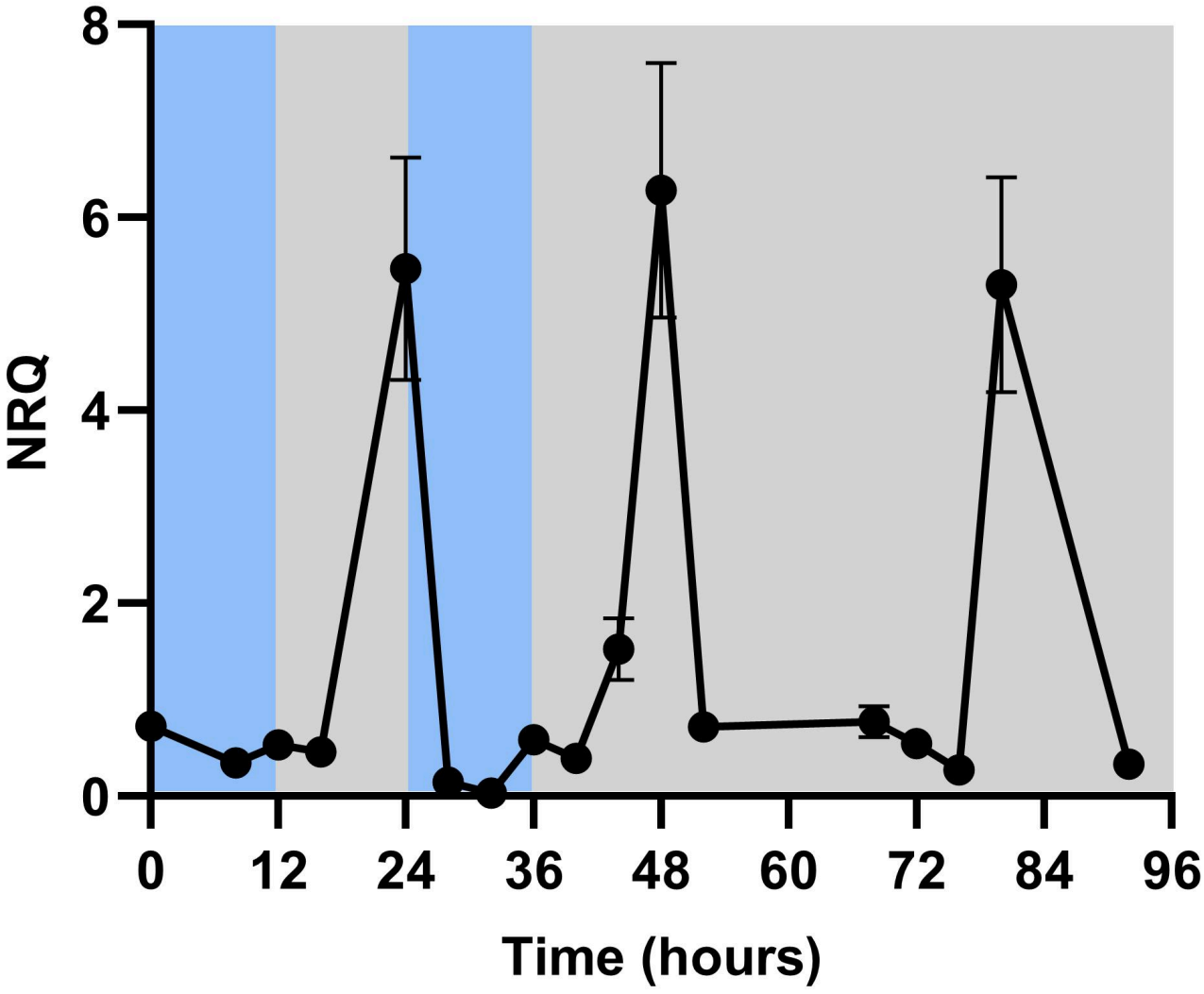

Figure 1

Rhythmic oscillations in *blsA* expression. *blsA* expression levels determined by RT-qPCR in *A. baumannii* V15 wild-type cultured under blue light (L) and in the dark (D) in 12L:12D photoperiod cycles for 5 days, and then released to constant darkness at 23ºC. The data shown are mean ± SE of normalized relative quantities (NRQs) [34] calculated from transcript levels measured in samples grown in LB broth, in at least two biological replicates. Gray and white backgrounds correspond to dark and blue light incubations, respectively.

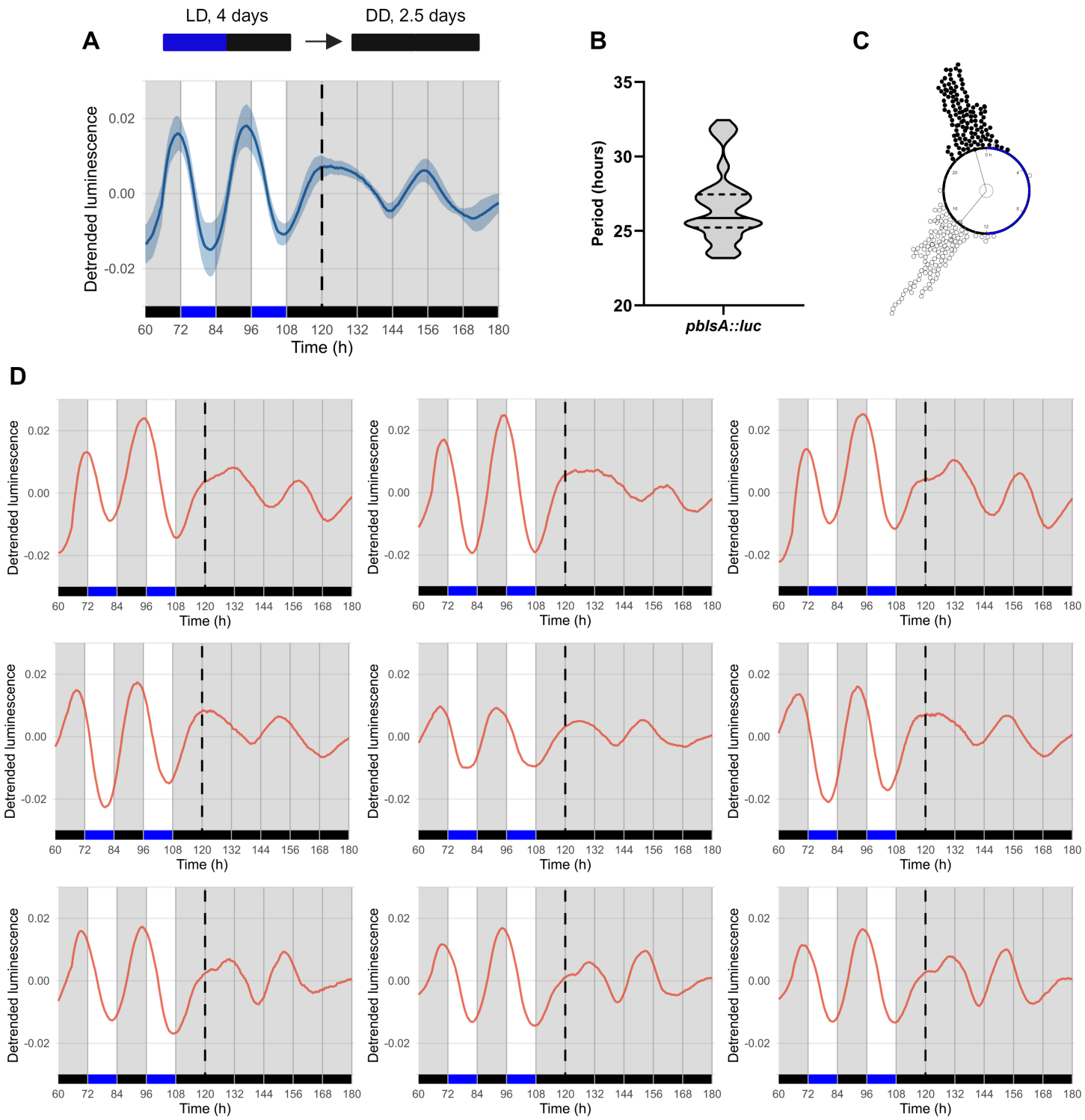

Figure 2

blsA–driven luminescence is rhythmic under entrained and FR conditions. (A) Average reporter activity of *A. baumannii* V15 pLPV1Z-*pblsA::luc* clone 1 under blue light/dark (bLD) and FR conditions. This strain was incubated in bLD cycles for 4 days and then released to DD. Black/blue bars indicate dark/light periods. Luminescence signals are shown as mean ± SEM. (B) Average endogenous period of rhythmic populations of *A. baumannii* V15 pLPV1Z-*pblsA::luc*clone 1 (26 ± 2.4 h, n=123). (C) Rayleigh plots

showing the phase of the bioluminescent peak under cyclic conditions (bLD, black dots) and the first bioluminescent peak on the first day of release to FR (DD, white dots) for the rhythmic population (bLD: 22.9 ± 0.8 h, n=123; R=0.98 and DD: 14.6 ± 1.5 h, n=123; R=0.92). Lines represent the average peak phase of *pblsA::luc* expression (mean vectors for the circular distributions) of each group. The length of the vector represents the strength of the phase clustering while the angle of the vector represents the mean phase. Individual data points are plotted outside the circle. The central circle represents the threshold for $p = 0.05$. (D) Representative single traces of luciferase activity rhythms from single wells. Bacteria were grown at a constant temperature of 23°C. (B) and (C) analysis included three biological replicates. Number of wells included: 123. One representative biological replicate is shown in (A).

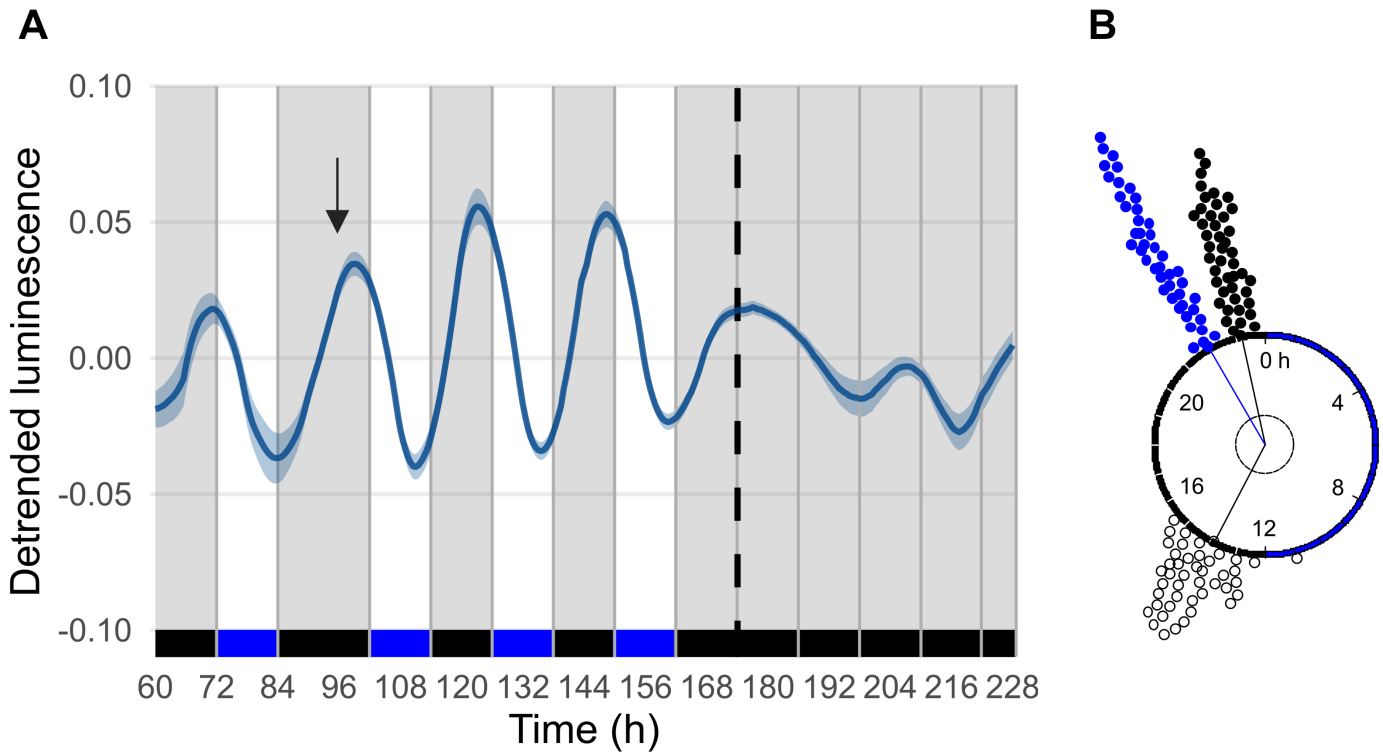

Figure 3

Luminescence rhythms respond to a phase shift in the photic periodic conditions. (A) Average population luminescence rhythms after a 6-h phase shift (n = 44) in *A. baumannii* V15 pLPV1Z-*pblsA::luc*clone 1. The arrow indicates the time of the phase shift. Luminescence signals are shown as mean ± SEM. (B) Rayleigh plots of bLD 1 (black dots: 23.2 ± 0.2 h, n = 44; R = 0.99), bLD 2 (blue dots: 22 ± 0.1 h, n = 44; R = 0.99), and DD (white dots: 13.8 ± 0.8 h, n = 44; R = 0.79). Rayleigh test, $P < 0.001$. Bacteria were grown at a constant temperature of 23°C.

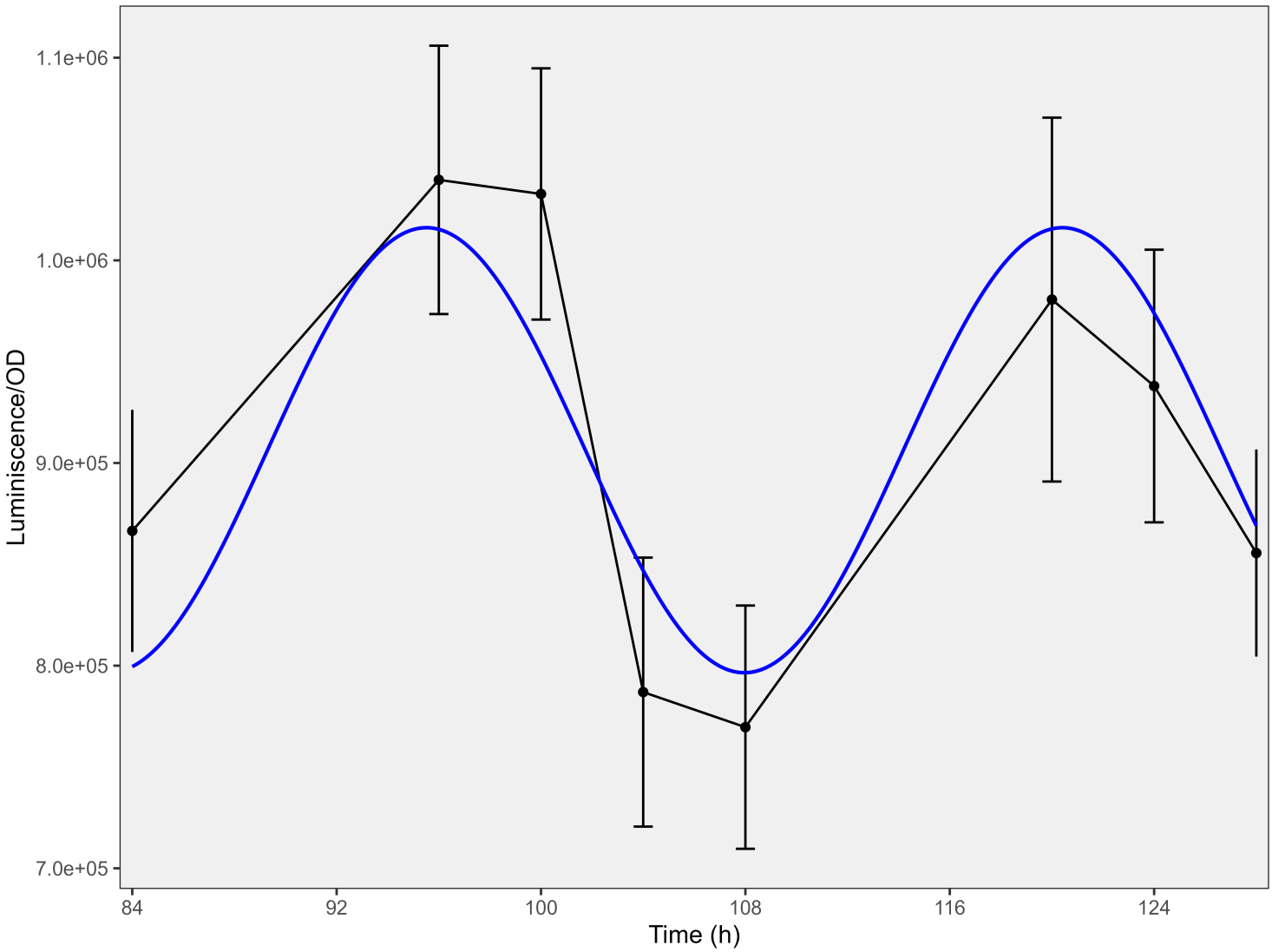

Figure 4

Endogenous *A. baumannii*'s rhythms. *A. baumannii* V15 pLPV1Z-*pblsA::luc*clone 1 was grown stagnantly at 23°C for 4-6 days under constant dark conditions. Discrete bioluminescent measurements were analyzed using Cosinor. Shown is a representative result from 4 independent experiments.

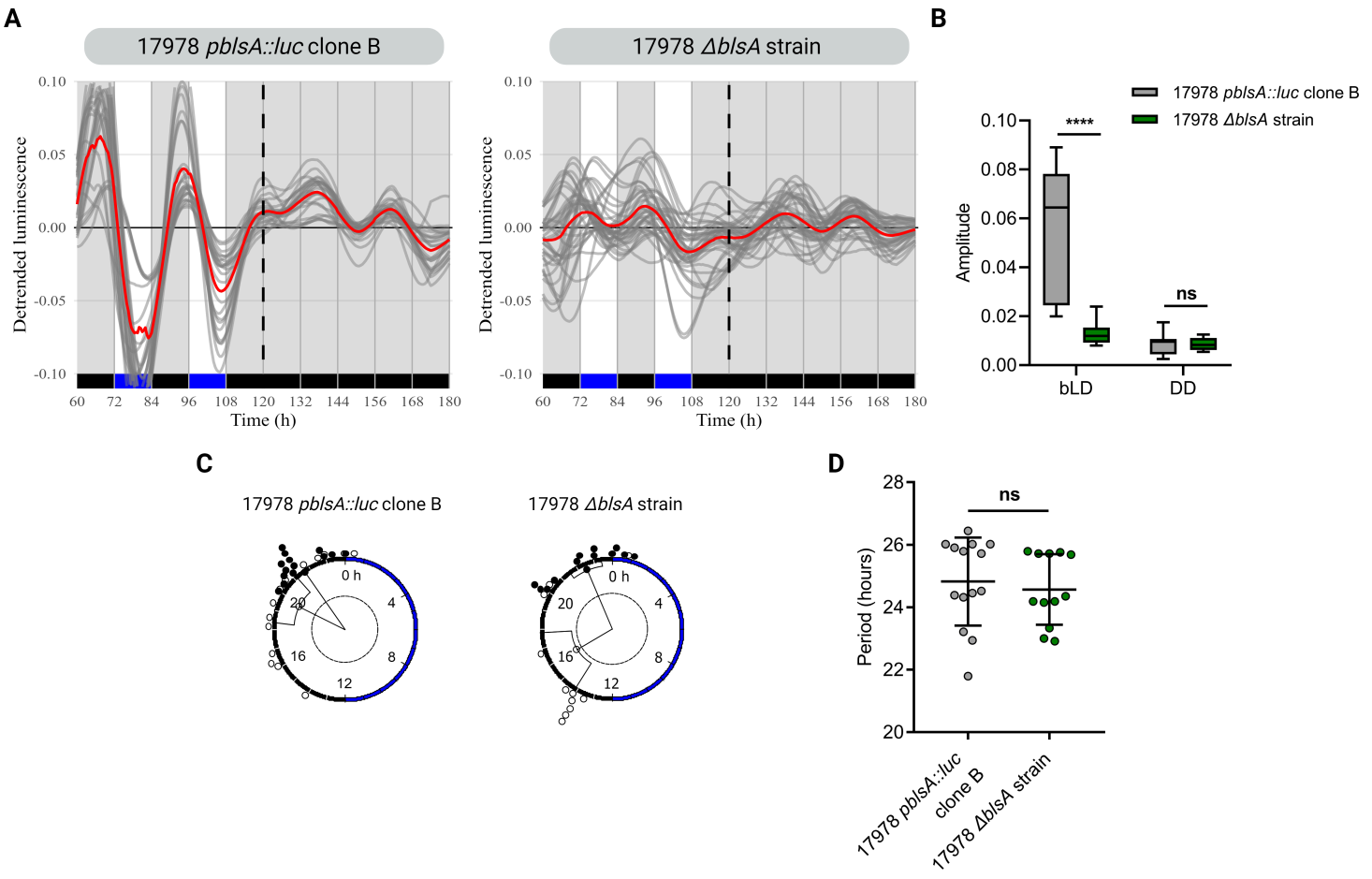

Figure 5

Blue light synchronization of bioluminescent rhythms requires the photoreceptor BlsA. (A) Reporter activity of *A. baumannii* ATCC 17978 strain (n = 23) and isogenic *ΔblsA* mutant (n = 36) containing pLPV1Z-*pblsA::luc* under blue light/dark (bLD) and FR conditions. The strains were incubated in bLD cycles for 4 days and then released to DD. Black/blue bars indicate dark/light periods. Luminescence signals are shown as mean ± SEM in red line and all the individual wells are represented in the grey lines. (B) Average amplitude of the luminescence rhythm of rhythmic populations of *A. baumannii* ATCC 17978 strain and *ΔblsA*mutant. Two-way ANOVA followed by Sidak's multiple comparisons test. (C) Rayleigh plots showing the acrophases of the bioluminescent signal in bLD, (black dots) and the first bioluminescent peak on the first day of release to FR (DD, white dots); n = 14 for control and n = 12 for mutant. The remaining populations were arrhythmic under FR conditions. Lines represent the average peak phase of *pblsA::luc*expression (mean vectors for the circular distributions) of each group. The length of the vector represents the strength of the phase clustering while the angle of the vector represents the mean phase. Individual data points are plotted outside the circle. The central circle represents the threshold for *p*= 0.05. (D) Average endogenous period of rhythmic populations of *A. baumannii* ATCC 17978 strain (24.8 ± 1.4 h, n=14) and *ΔblsA* mutant (24.5 ± 1.1 h, n=12). Bacteria were grown at a constant temperature of 23°C.

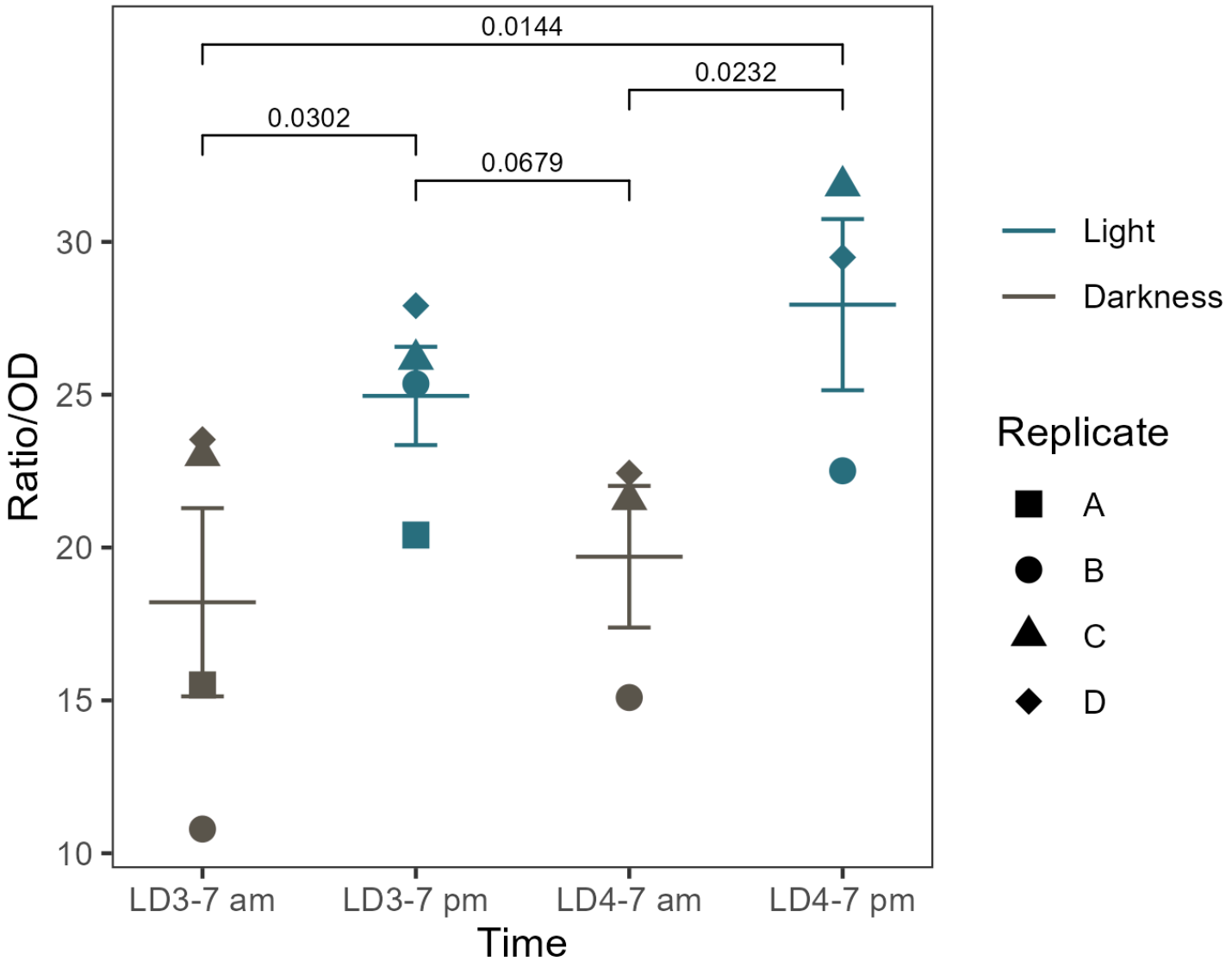

Figure 6

β-lactamase activity fluctuates along the day. *A. baumannii* Ab825 cells were grown overnight in LB at 37 ºC in the dark, and then inoculated in fresh new LB media at a DO_{600}= 0.05. The bacteria were then grown under 12L:12D photoperiod for 4 days at 23ºC. At 7 am and 7 pm of the 3rd and 4th days (LD3 and LD4), samples were retrieved and processed using a nitrocephin-based colorimetric method for β-lactamase activity detection following the manufacturer recommendations (Amplite Colorimetric Beta-Lactamase Activity, AAT Bioquest). The reactions were incubated at room temperature, with the plate protected from light, and after 60 minutes absorbances at 490, 380, and 600 nm were determined using in a microplate reader (Bio Tek Instruments EPOCH2T). 490/380 ratios were calculated using these values and normalized to the OD_{600} corresponding to the time point for each sample. Colored lines in each time point show mean values±standard deviation. Segments in black indicate p-values of significant post-hoc comparisons based on a mixed model with random replicate effect.

Supplementary Files

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- SupplementaryFigure1.png
- Supplementaryfigure2.tif