Blue Light Directly Modulates the Quorum Network in the Human Pathogen *Acinetobacter baumannii*

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Abstract

Quorum sensing modulates bacterial collective behaviors including biofilm formation, motility and virulence in the important human pathogen *Acinetobacter baumannii*. Disruption of quorum sensing has emerged as a promising strategy with important therapeutic potential. In this work, we show that light modulates the production of secreted molecules that complement motility in the acyl-homoserine lactone (AHL) synthase mutant, *abaI*, at environmental temperatures. Also, AHLs were produced in higher levels in the dark than under blue light at environmental temperatures, a response that depends on AbaI and on the photoreceptor BlsA. BlsA interacts with the transcriptional regulator AbaR in the dark at environmental temperatures, inducing *abaI* expression. Under blue light, BlsA does not interact with AbaR, but induces expression of the lactonase *aidA* and quorum quenching, consistently with lack of motility at this condition. At temperatures found in warm-blooded hosts, the production of AHLs, quorum quenching as well as *abaI* and *aidA* expression were also modulated by light in a BlsA-independent manner. Finally, AbaI reduces *A. baumannii*'s ability to kill *C. albicans* only in the dark both at environmental as well as at temperatures found in warm-blooded hosts. The overall data indicate that light directly modulates quorum network in *A. baumannii*.

Introduction

Acinetobacter baumannii has been recognized by the World Health Organization (WHO) as one of the most threatening bacterial pathogens deserving urgent action [1]. The global menace from this pathogen arises from its high ability to develop antibiotic resistance as well as its outstanding ability to persist in the environment, which lead to the rapid emergence and spread of multidrug-resistant clinical isolates [2,3]. This makes the need for understanding the mechanisms of resistance and virulence critical. Quorum sensing (QS) has been shown to modulate bacterial collective behaviors such as biofilm formation, motility, virulence, and even drug resistance mechanisms in a number of bacteria including *A. baumannii* [4,5,6,7]. Lately, disruption of QS has emerged as an anti-virulence strategy with important therapeutic potential [8].

Quorum sensing is a cell-cell communication system employed by bacteria to coordinate the expression of specific genes as a function of population density. In most Gram-negative bacteria, QS is mediated via the synthesis, release and detection of diffusible signaling molecules such as the N-acyl-homoserine lactones (AHLs) [9]. *A. baumannii* and related pathogenic *Acinetobacter* spp. possess a canonical Gram-negative LuxR/LuxI QS system, consisting of an AHL synthase (AbaI) and a transcriptional regulator (AbaR) that is activated on binding an AHL, leading ultimately to diverse cellular responses [10]. AbaR bound to AHL triggers the production of more AHLs in a positive feedback loop manner. The complete genome sequencing of *A. baummannii* ATCC 17978 revealed that AbaI and acetyl transferases may be the sole participants for the production of AHLs with varying chemical structures [5]. Recently, the product of the *abaM* gene, which is located between the *abaR* and *abaI* genes in the *A. baumannii* chromosome, has been shown to play a central role in QS negative regulation [11]. Quorum quenching (QQ) refers to all

processes involved in the disturbance of QS [12]. QQ molecular actors are diverse in nature (enzymes, chemical compounds), mode of action (QS-signal cleavage, competitive inhibition, etc.) and targets, as all main steps of the QS pathway that are synthesis, diffusion, accumulation and perception of the QS signals may be affected [13].

Some years ago, we have recognized a new aspect of *A. baumannii*'s physiology: its ability to perceive light and respond to this stimuli modulating different traits related to persistence at environmental temperatures [14]. In fact, light modulates biofilm formation, motility, killing of competitors, phenylacetic acid, trehalose and acetoin metabolisms, iron uptake, antioxidant enzymes production, antibiotic susceptibility and tolerance to antibiotics, at environmental temperatures [14–21]. Regulation by light of many of these traits depends on the photoreceptor BlsA, which is operative in the 18–24ºC low-moderate temperature range [14–16]. We have shown that BlsA interacts with and antagonizes the action of Fur or AcoN transcriptional repressors, allowing expression of their regulated genes only in the dark or in the presence of blue light, respectively [18,20,21]. More recently, we have provided evidence indicating that ESKAPE pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, also perceive and respond to light at temperatures found in warm-blooded hosts, regulating virulence in an epithelial infection model as well as important pathogenicity determinants, which could have implications in human infections [22].

In Tuttobene *et al.* 2019, we observed that both quorum and light integrate signals into acetoin catabolism through the AcoN transcriptional regulator in *A. baumannii* [21]. This connection prompted us to systematically explore the possible relation between light and quorum in *A. baumannii*. We decided to focus on two community behaviors such as motility and biofilm formation shown to depend on quorum sensing, and which are also modulated by light [14,16]. Our results show that there is a fine tuning of quorum sensing vs. quenching activities dictated by modulation of the expression of AHL synthases and lactonases by light, integrating also a temperature signal, which results in the differential production of AHLs in response to illumination and temperature. Overall, in this work we provide evidence indicating that light directly modulates the quorum network in *A. baumannii*.

Results

AbaI is a direct or indirect component of the light signaling cascade in _A. baumannii_ at environmental temperatures. *A. baumannii* ATCC 17978 moves covering the whole plate in the dark, while it's motility is inhibited in the presence of light at 23ºC (Fig. 1A) [14]. The mutant in the only traditional photoreceptor encoded in the *A. baumannii* genome, *blsA*, lost photoregulation at this temperature, as motility covered the whole plates under both illumination conditions (Fig. 1A) [14]. Several studies have shown that *A. baumannii abaI* mutants are deficient in motility and biofilm formation [5,10,23,24]. Figure 1A shows that in the *ΔabaI* mutant motility was abolished both under blue light and in the dark at 23ºC, as also was motility in the *ΔabaI* mutant harboring the pWH1266 plasmid. In contrast, the *ΔabaI* mutant containing plasmid pWHAbaI, which expresses a wild type copy of *abaI* directed by its own promoter, rescued the

wild type phenotype resulting in motility only in the dark but not under blue light (Fig. 1A), integrating thus a light signal.

The results presented here show that AbaI is required for motility at environmental temperatures, and that it is a direct or indirect component of the light signaling cascade.

Light modulates the production of AHLs in motility and biofilms through BlsA and AbaI in *A. baumannii* at moderate temperatures. To study if quorum sensing is modulated by light in *A. baumannii*, we first evaluated the effect of light in AHLs production on quorum-dependent processes by using different bacterial biosensors. AHL biosensors are bacterial strains mutants in the gene coding for the AHLs synthase, in which the only possibility to activate the quorum cascade is upon complementation with exogenous AHLs, resulting in a clearly evident phenotype such as pigment production.

For this purpose, filtered sterilized supernatants were generated from cultures recovered from motility plates incubated under blue light or in the dark at 23ºC. The amounts of supernatants were normalized to bacterial optical density OD_{660} = 1.5 (see Methods for details), and then located in the center wells of biosensor-inoculated LB plates.

The *C. subtsugae* CV026 biosensor produces the violet pigment violacein in the presence of exogenous short chain N-acyl AHLs, from C_4 to C_8 [25]. Figure 2 shows that the supernatant recovered from ATCC 17978 motility plates incubated in the dark stimulated significantly the production of violacein in *C. subtsugae* CV026, approximately 65% relative to the positive control, indicating thus the presence of AHLs in this condition (Fig. 2A and B). In contrast, the absence of pigment in the case of the supernatant recovered from ATCC 17978 motility plates incubated under blue light, reflected the absence of detectable amounts of AHLs in this condition (Fig. 2A and B). The *ΔblsA* mutant produced AHLs both when incubated under blue light or in the dark, as similar amounts of violacein were produced at both conditions (approximately 65 and 62% relative to the positive control, respectively) (Fig. 2A and B); as also did the *ΔblsA* mutant harboring the empty pWH1266 plasmid (Fig. 2A and B). On the contrary, the *ΔblsA* mutant harboring plasmid pWHBlsA, which expresses a wild type copy of the *blsA* gene directed by its own promoter, rescued the wild type phenotype. In this case, the production of violacein was significantly stimulated only when supernatants recovered from motility plates incubated in the dark but not under blue light were added to *C. subtsugae* CV026 cultures (Fig. 2A and B). It is noteworthy that incubation of *C. subtsugae* CV026 with the supernatant recovered from *ΔblsA* pWHBlsA motility plates incubated in the dark, resulted in higher violacein production levels (100% relative to the positive control) than in the case of supernatants recovered from the wild type strain incubated in the dark (approximately 65% relative to the positive control). This effect could be explained considering the extra *blsA* gene doses in the former, as has already been observed before [14]. The supernatants obtained from *ΔabaI* motility plates incubated under blue light or in the dark at 23ºC both failed to induce violacein production, as reflected in the absence of violet color (Fig. 2A and B). This indicates absence of detectable amounts of AHLs production in the *ΔabaI* mutant, as expected. The *ΔabaI* mutant harboring the empty pWH1266 plasmid behaved as the *ΔabaI* mutant (Fig. 2A and B). The *ΔabaI* mutant harboring pWHAbaI, in contrast,

rescued the wild type phenotype (Fig. 2A and B), i.e., only supernatant recovered from *ΔabaI* pWHAbaI motility plates incubated in the dark significantly stimulated the production of violacein in *C. subtsugae* CV026, but not that recovered from plates incubated under blue light. Again, the supernatant recovered from *ΔabaI* pWH*abaI* motility plates incubated in the dark, resulted in higher violacein production levels (100% respect to the positive control) than in the case of those recovered from the wild type strain incubated in the dark (approximately 65% relative to the positive control). This effect could be explained considering the extra *abaI* gene doses in the former. Overall, these results thus show that the production of short-chain AHLs is modulated by light at moderate temperatures in *A. baumannii* ATCC 17978, in a BlsA and AbaI-dependent manner.

Similar experiments performed using the *C. violaceum* VIR07 biosensor, which synthesizes violacein in the presence of long-chain AHLs from C_{10} to C_{16}, produced similar pattern as that observed for C. *subtsugae* CV026 (Fig. 2A and B), indicating that the production of long-chain AHLs is also regulated by light in motility in *A. baumannii* at moderate temperatures, in a BlsA and AbaI-dependent manner (Fig. 2C and D).

We also assayed the effect of light on AHL production in another quorum-dependent phenotype, biofilm formation. For this purpose, filtered sterilized supernatants were generated from cultures recovered from biofilm tubes incubated under blue light or in the dark at 23ºC. The amounts of supernatants were normalized to bacterial optical density OD_{660} = 0.5 (see Methods for details), and then located on wells in the center of biosensor-inoculated LB plates.

As previously described and as shown in Fig. 1B, biofilm formation is modulated by light in *A. baumannii* at environmental temperatures, in a BlsA-dependent manner. In particular, biofilms are significantly produced in the dark while inhibited in the presence of light in ATCC 17978. The photorregulation is lost in the *blsA* mutant, and the wild type phenotype is rescued in the *blsA* complementing strain (Fig. 1B). Biofilm is not produced in the *abaI* mutant, while the *abaI* complementing strain rescued the wild type phenotype, indicating that photoregulation is also dependent on AbaI (Fig. 1B).

Supplementary Fig. 1 shows that supernatants recovered from ATCC 17978 biofilms incubated in the dark significantly stimulated the production of violacein in *C. subtsugae* CV026 (approximately 50% relative to the positive control), indicating the presence of AHLs in this condition (Supplementary Fig. 1A and B). In contrast, the absence of pigment in the case of supernatants recovered from ATCC 17978 biofilms incubated under blue light, reflected the absence of detectable amounts of AHLs in this condition (Supplementary Fig. 1A and B). The *ΔblsA* mutant produced AHLs both when biofilm tubes were incubated under blue light or in the dark, as similar amounts of violacein were produced at both conditions (approximately 50% relative to the positive control) (Supplementary Fig. 1A and B); as also did the *ΔblsA* mutant harboring the empty pWH1266 plasmid (Supplementary Fig. 1A and B). On the contrary, the *ΔblsA* mutant harboring plasmid pWHBlsA rescued the wild type phenotype. In fact, the production of violacein was significantly stimulated only when supernatants recovered from biofilms incubated in the dark but not under blue light were added to *C. subtsugae* CV026 cultures (Supplementary Fig. 1A and B).

It is noteworthy that incubation of *C. subtsugae* CV026 with the supernatant recovered from *ΔblsA* pWHBlsA biofilms incubated in the dark, resulted in higher violacein production levels (100% relative to the positive control) than in the case of supernatants recovered from the wild type strain incubated in the dark (approximately 50% relative to the positive control). This effect can be explained considering the extra *blsA* gene doses in the former, as has already been observed above for motility. The supernatants obtained from *ΔabaI* biofilms incubated under blue light or in the dark at 23ºC failed to induce violacein production, as reflected in the absence of violet color (Supplementary Fig. 1A and B). This indicates absence of detectable amounts of AHLs production in the *ΔabaI* mutant, as expected. The *ΔabaI* mutant harboring the empty pWH1266 plasmid behaved as the *ΔabaI* mutant (Supplementary Fig. 1A and B). The *ΔabaI* mutant harboring pWH*abaI*, in contrast, rescued the wild type phenotype (Supplementary Fig. 1A and B), i.e., only supernatant recovered from *ΔabaI* pWHAbaI biofilms incubated in the dark significantly stimulated the production of violacein in *C. subtsugae* CV026, but not that recovered from plates incubated under blue light. Again, the supernatant recovered from *ΔabaI* pWHAbaI motility plates incubated in the dark, resulted in higher violacein production levels (100% relative to the positive control) than in the case of supernatants recovered from the wild type strain incubated in the dark (approximately 50% relative to the positive control). Overall, these results thus show that the production of short chain AHLs is modulated by light in biofilms at moderate temperatures in *A. baumannii* ATCC 17978, in a BlsA and AbaI-dependent manner.

Similar experiments performed using the long-chain AHLs biosensor *C. violaceum* VIR07, produced similar pattern as that observed for C. *subtsugae* CV026 (Supplementary Fig. 1A and B), indicating that the production of long chain AHLs is regulated by light in biofilms in *A. baumannii*, in a BlsA and AbaI-dependent manner at moderate temperatures (Supplementary Fig. 1C and D).

Finally, AHL production (Figs. 2 and Supplementary Fig. 1) follows the motility as well as the biofilm formation pattern (Fig. 1A and B).

Light modulates *abaI* expression at 23ºC through the BlsA photoreceptor. To get insights into the molecular mechanism of light modulation of quorum sensing, we studied the expression of *abaI* in cells recovered from motility plates incubated under blue light or in the dark at 23ºC. Figure 1C shows that expression of *abaI* is approximately 38 fold higher in the dark than in the presence of light, indicating that it is modulated by light in ATCC 17978. In the *Δbls*A mutant, *abaI* expression levels were similar between light and dark conditions, and comparable to wild type levels in the presence of light (Fig. 1C), showing loss of photoregulation. As expected, expression of *abaI* in the *ΔabaI* mutant was null or negligible (Fig. 1C). The overall results show that the expression of *abaI* is modulated by light, being induced in the dark at 23ºC through the BlsA photoreceptor. This result is consistent with the higher production of AHLs in the dark in ATCC 17978.

ATCC 17978 produces diffusible molecules able to restore *ΔabaI* motility defect. We studied here whether *A. baumannii* is able to produce diffusible molecules capable of complementing the lack of motility shown by the AHL synthase mutant, *ΔabaI.* As shown in Fig. 3A and B, the filtered sterilized supernatants

recovered from motility plates inoculated with ATCC 17978 and incubated both under blue light and in the dark at 23ºC were able to stimulate *ΔabaI* motility, but only when the *ΔabaI*-inoculated plates were incubated in the dark and not under blue light (Fig. 3A and B). Similar results were obtained for the *ΔblsA* mutant (Fig. 3A and B). These results show that *A. baumannii* produces diffusible molecules able to restore *ΔabaI* motility defect. Most likely these molecules are AHLs, as they complement the AHL synthase mutant phenotype. Interestingly, the ability of ATCC 17978 supernatants to complement *abaI* mutant was higher in the dark than under blue light (100% vs. 60% plate coverage, respectively)(Fig. 3), which is consistent with the higher production of AHLs detected in the dark both for the *C. subtsugae* CV026 as well as the *C. violaceum* VIR07 biosensors (Fig. 2 and Supplementary Fig. 1). On the contrary, the ability of the *blsA* mutant supernatants to complement the *abaI* mutant was similar disregarding whether the cells were incubated under blue light or in the dark (100% plate coverage for both conditions) (Fig. 3), also consistently with results obtained using the biosensors indicating loss of photoregulation of AHL production in this mutant (Fig. 2 and Supplementary Fig. 1). Finally, these results suggest that the presence of some AHLs-inactivating activity in *ΔabaI* in the presence of light, as the stimulation of motility observed in dark conditions was completely abolished in this condition.

Light stimulates quorum quenching activity at moderate temperatures in *A. baumannii.* As another approach to study the effect of light on the production of quorum quenching molecules at moderate temperatures, we evaluated whether the supernatants of sonicated cells recovered from motility plates incubated under blue light or in the dark presented quorum quenching activity. For this purpose, these supernatants were incubated in the presence of 2 µg of C8-AHL for 6 hs, and then the mixture was loaded on a central well generated in a *C. subtsugae* CV026-inoculated LB plate. The extent of inhibition of violacein production compared to the control provides an indication of quorum quenching activity.

As shown before, the addition of 2 µg of commercial C8-AHL to the central well of *C. subtsugae* CV026-inoculated LB plates stimulated the production of violacein in the whole plate (Fig. 4A and B). Interestingly, when this fixed amount of standard was incubated with the supernatant of the sonicated wild type cells recovered from motility plates incubated under blue light, violacein production was less stimulated than when they were incubated in the dark (approximately 20% vs. 47% relative to the positive control, respectively) (Fig. 4A and B). In contrast, when the standard was incubated with supernatants recovered from sonicated *ΔblsA* mutant cells incubated either under blue light or in the dark, similar capability to inhibit violacein production in *C. subtsugae* CV026 was observed, which was similar to that of the wild type incubated in the dark; showing thus loss of photoregulation of quorum quenching activity (Fig. 4A and B). Finally, when supernatants obtained from sonicated *ΔabaI* cells recovered from motility plates incubated under blue light at 23ºC were used, no violacein production was observed indicating that the standard was completely degraded (Fig. 4A and B). On the contrary, when the standard was incubated with supernatants of sonicated *ΔabaI* pWH*abaI* cells recovered from motility plates incubated in the dark at 23º, *C. subtsugae* CV026 produced an important violacein halo comparable to that of the wild type in the dark (approximately 50% relative to the positive control), while no violacein was detected in the presence of blue light. These results thus indicate much higher quorum quenching activity under blue light than in the dark in this strain, much higher even than in the wild type strain (Fig. 4A and B). Overall,

we show here that the production of AHL-inactivating molecules is modulated by light, in a BlsA-dependent manner.

Similar experiments performed using the *C. violaceum* VIR07 biosensor and C10-AHL as standard, produced similar patters as above (Fig. 4C and D), indicating that quorum quenching activity affects long-chain AHLs as well. The fact that quorum quenching activity was observed in sonicated supernatants strongly suggest the presence of non-secreted lactonase/s.

AidA expression is modulated by light at 23ºC through the BlsA photoreceptor.

Given that our data provide evidence indicating modulation by light of quorum quenching activity, we next decided to analyze by qRT-PCR whether expression of the gene coding for a recently described lactonase activity capable of degrading AHLs involved in QS, *aidA*, is responsive to light [26]. AidA has been proposed to degrade 3-oxo-C12-HSL, however, its activity has not been characterized against other AHL. It is possible though that as a lactonase it is able to degrade different types of AHLs [26].

Our results show that *aidA* expression is approximately 7 folds higher when ATCC 17978 cells were recovered from motility plates incubated under blue light compared to darkness (Fig. 1D). The *ΔabaI* mutant behaved similarly as the wild type strain, showing differential expression of *aidA* in response to light, despite expression levels were much higher both under blue light and in the dark (Fig. 1D). The observed photoregulation depends on BlsA, since the *ΔblsA* mutant was basal and similar under blue light and in the dark (Fig. 1D).

The results obtained here show that expression of *aidA* gene in ATCC 17978 and in the *ΔabaI* mutant is modulated by light through the BlsA photoreceptor at environmental temperatures. This result is consistent with the modulation by light of quorum quenching activity shown using biosensors (Fig. 4).

AbaI is involved in modulation by light of *A. baumannii* ATCC 17978 ability to kill *C. albicans tup1* at environmental temperatures. To study the role of quorum sensing and light in the ability of *A. baumannii* to kill *C. albicans* at moderate temperatures, ATCC 17978 or its isogenic *ΔabaI* mutant and derived strains were co-incubated with *C. albicans tup1* at 23ºC, and the survival of *C. albicans* was recorded as described in [14]. For killing assays, *C. albicans tup1* mutants were used, as it was previously shown that *A. baumannii* is able to attach to and kill *tup1* filaments but not the parental SC5314 yeast cells [27].

C. albicans tup1 filaments recovery was significantly lower when co-incubated with *A. baumannii* ATCC 17978 under blue light than in the dark, showing that *A. baumannii* is more virulent against *C. albicans tup1* under illumination conditions (Fig. 5A), which is consistent with previous results [14]. Conversely, the number of *tup1* mutant filaments recovered when co-incubated with the *ΔabaI* mutant was similar between light and darkness, and also similar to that recovered when co-incubated with the wild type strain under blue light (Fig. 5A). Thus, the *ΔabaI* mutant lost the ability to photoregulate killing of *tup1* mutant filaments. The *ΔabaI* mutant harboring the empty pWH1266 plasmid behaved as the *ΔabaI* mutant (Fig. 5B). In contrast, the *ΔabaI* mutant harboring plasmid pWHAbaI restored the wild type

phenotype showing increased virulence under blue light (Fig. 5B). As controls, no differences between light and darkness were detected in the number of *tup1*filaments recovered when incubated without bacteria in LB or in preconditioned media (PCM) (Fig. 5C and D, respectively), indicating that growth is not affected by blue light.

The overall results show that AbaI is involved in modulation by light of *A. baumannii*'s virulence when tested using the killing of *C. albicans tup1* mutant filaments model. AbaI is necessary for the lower virulence exhibited by *A. baumannii* in the dark (Fig. 5A and B), while the presence of BlsA, in turn, is involved in stimulation of virulence in the presence of light [14].

BlsA interacts with the regulator AbaR in the dark at moderate temperatures in *A. baumannii*. To study whether BlsA interacts with AbaR, we performed yeast two-hybrid experiments using an adapted version of ProQuestTM Two-Hybrid System [20,21]. Strain Mav 203, which is included as host yeast in the system, harbors three reporter genes with different promoters: *lacZ* and HIS3 and URA3. His and URA3 solve the histidine and uracile MAV203 auxotrophies upon expression. Then, if BlsA and AbaR do interact, it would be expected the appearance of blue color as well as growth in the absence of histidine or uracil. pGAD-T7Gw and pGBK-T7Gw plasmids have been adapted to express *blsA* and *abaR*, as fusions to GAL4 DNA binding domain (DB) or activation domain (AD). Self-activation controls (pGAD-T7Gw and pGBK-T7Gw empty vectors) as well as different strength interaction controls (A–E) were also included. BlsA was previously shown to interact with different partners in an illumination and temperature-dependent manner [20,21]. Figure 1E shows results of Y2H assay experiments performed under different illumination and temperature conditions. BlsA-AbaR interaction was observed as the appearance of blue color and growth in SC defined media without the addition of histidine or uracil only at 23 ºC in the dark (Fig. 1E). Both pGAD*blsA*/pGBK*abaR* and pGAD*abaR*/pGBK*blsA* combinations generated positive signals, indicating that the interactions occurred independently of the host vector (Fig. 1E). Also, absence of self-activation of each protein fused to DB or AD: (pGAD-T7/pGBK*blsA* or pGBK*abaR*) or (pGBK-T7/pGAD*blsA* or pGAD*abaR*) was verified in the corresponding controls (Fig. 1E). On the contrary, no interactions were observed for AbaR and BlsA for any of the reporters tested at 23 ºC in the presence of blue light, despite interaction controls behaved as expected (Fig. 1E). Altogether, the data indicate that BlsA interacts with AbaR only in the dark at 23ºC, in a light-dependent manner.

At higher temperatures such as 30ºC, null or negligible BlsA–AbaR interactions were observed (Fig. 1E), either in the dark nor under blue light.

Light modulates the production of AHLs in *A. baumannii* at 37°C. As has been previously reported, motility is not photoregulated in *A. baumannii* ATCC 17978 at 37ºC [14] (Supplementary Fig. 2), and neither is biofilm formation in glass (not shown). However, the production of violacein was found to be enhanced approximately 2.3 folds when *C. subtsugae* CV026 was supplemented with supernatants recovered from motility plates incubated under blue light vs. in the dark at 37ºC, thus indicating the presence of higher amounts of AHLs under blue light (Fig. 6A and B). Supernatants recovered from the *ΔblsA* mutant behaved as the wild type, i.e., generated enhanced violacein production under blue light compared to dark

conditions (Fig. 6A and B). The *abaI* mutant as well as the *abaI* mutant harboring the empty pWH1266 plasmid, did not produce violacein, neither under blue light nor in the dark (Fig. 6A and B). This is consistent with the absence of AHLs expected in the AHL synthase mutant. On the contrary, expression of *abaI* from plasmid pWH*abaI* in the *abaI* mutant background restored violacein production. Similarly to the wild type, violacein production was higher under blue light compared to dark conditions (100% vs. approximately 35% relative to the positive control). These results show that the production of short-chain AHLs production is regulated by light in *A. baumannii* at 37ºC, in a BlsA-independent but AbaI-dependent manner.

Similar experiments performed using the long-chain AHLs biosensor *C. violaceum* VIR07, produced similar pattern as that observed for *C. subtsugae* CV026 (Fig. 6A and B), indicating that the production of long-chain AHLs is regulated by light in motility in *A. baumannii* at 37ºC, in a BlsA-independent AbaI-dependent manner (Fig. 6C and D).

Finally, filtered-sterilized supernatants recovered from biofilm tubes incubated under blue light or in the dark at 37ºC were also used to supplement the *C. subtsugae* CV026 and *C. violaceum* VIR07 biosensors (Supplementary Fig. 3). Again, violacein production was found to be higher under blue light than in the dark in the ATCC 17978 wild type strain. Also, the derivative strains showed a similar pattern as that observed for supernatants recovered from motility plates (Fig. 6). Overall, Supplementary Fig. 3 indicates that the production of both short as well as long-chain AHLs is regulated by light in biofilms in *A. baumannii* at 37ºC, with increased AHL production under blue light compared to dark conditions, in a BlsA-independent AbaI-dependent manner.

It should be noted that at 37ºC the production of violacein is higher under blue light than in the dark both for motility as well as biofilm formaion, while the opposite occurs at 23ºC, i.e., violacein production is higher in the dark than under blue light. In addition, the magnitude of the light-dark difference is much higher at 23ºC than at 37ºC.

Quorum quenching activity is stimulated in the dark at 37ºC in *A. baumannii.* We evaluated here whether the supernatants of sonicated cells recovered from motility plates incubated under blue light or in the dark at 37ºC presented quorum quenching activity. For this purpose, these supernatants were incubated in the presence of 2 µg of standard for 6 hs, and then the mixture was then loaded on a central well generated in a biosensor-inoculated LB plate. The extent of inhibition of violacein production compared to the control without supernatant provides an indication of quorum quenching activity.

When the *C. subtsugae* CV026 biosensor and the C8-AHL standard were used, quorum quenching activity was observed for supernatants incubated both under blue light and in the dark for all the strains studied, i.e. the wild type and the Δ*blsA* and Δ*abaI* mutants. Quorum quenching activity was only slightly higher in the dark than under blue light for all the strains included (approximately 75% vs. 55% relative to the positive control in all cases) (Fig. 7A and B). On the contrary, when the *C. violaceum* VIR07 biosensor and the C10-AHL standard were used, quorum quenching activity was much pronounced in the dark compared to illumination conditions for all the strains studied (approximately 50% vs. 10% relative to the

positive control in the wild type; while approximately 60% vs. 10% relative to the positive control in the *ΔblsA* and *ΔabaI* mutants) (Fig. 7C and D). These results show the presence of quorum quenching activity modulated by light in *A. baumannii* at 37ºC, in a BlsA and AbaI-independent manner.

***abaI* and *aidA* expression are modulated by light at 37ºC.** We studied next the expression of *abaI* in cells recovered from motility plates incubated under blue light or in the dark at 37ºC. Supplementary Fig. 2B shows that expression of *abaI* is approximately 1.5 folds higher under blue light than in the dark, indicating that it is modulated by light in ATCC 17978 at 37ºC. The *Δbls*A mutant behaved as the wild type strain, showing that modulation by light of *abaI* expression is independent of BlsA (Supplementary Fig. 2B). As expected, expression of *abaI* in the *ΔabaI* mutant was null or negligible (Supplementary Fig. 2B). The overall results show that the expression of *abaI* is modulated by light at 37ºC, being induced under blue light in a BlsA-independent manner. This result is consistent with the higher production of AHLs under blue light in ATCC 17978 at 37ºC. It is also worth mentioning the photorregulation of *abaI* expression and AHL production at 37ºC is opposite with respect to that observed 23ºC, as they are induced in the dark at 23ºC. In addition, the magnitude of the differences in *abaI* expression and AHL production between light and darkness were much higher at 23 than at 37ºC.

Interestingly, *aidA* expression pattern was inverse to that of *abaI*. In particular, *aidA* expression was induced approximately 2 folds in the dark compared to illumination conditions at 37ºC (Supplementary Fig. 2C). The *blsA* mutant behaved as the wild type, while the *abaI* mutant presented increased levels of expression both under blue light and in the dark than the wild type, while maintaining a difference of 1.5 folds between light and darkness (Supplementary Fig. 2C). This result is consistent with the higher quorum quenching activity detected in the dark in ATCC 17978 at 37ºC, as well as with the lower amounts of AHL observed in this condition.

Light modulates *A. baumannii*'s virulence against *C. albicans* at 37ºC through AbaI.

C. albicans tup1 filaments recovery was lower when co-incubated with *A. baumannii* ATCC 17978 under blue light than in the dark at 37ºC, showing that *A. baumannii* is more virulent against *C. albicans tup1* under blue light (Fig. 5E), as occurs at environmental temperatures (Fig. 5A). Conversely, the *ΔabaI* mutant showed an opposite pattern of photoregulation, with less *C. albicans tup1* filaments recovery in the dark than under blue light (Fig. 5E), indicating that the ability to kill *C. albicans tup1* is enhanced in the dark in this mutant. The *ΔabaI* mutant harboring the empty pWH1266 plasmid behaved as the *ΔabaI* mutant (Fig. 5F). In contrast, the *ΔabaI* mutant harboring plasmid pWHAbaI, which expresses a wild type copy of *abaI* directed by its own promoter, restored the wild type phenotype showing increased virulence under blue light (Fig. 5F). *ΔblsA* behaved similarly as the wild type, suggesting that it is not involved in this response to light at 37ºC (Fig. 5G and H). As controls, *C. albicans tup1* filaments recovery was similar between blue light and in the dark both in LB and in (PCM) (Fig. 5I and J, respectively), indicating that growth is not affected by blue light. From the above results, it follows that in the absence of AbaI the bacteria display a virulence mechanism modulated by light at 37ºC, in which virulence is enhanced in the dark instead than under blue light, as occurs in a wild type background. Overall, AbaI is involved in

modulation by light of *A. baumannii*'s virulence against *C. albicans tup1*at temperatures found in warm-blooded hosts.

Discussion

In this work, we provide strong evidence indicating that light directly modulates the quorum network in *A. baumannii*. We focused on motility and biofilms formation community responses, which are different bacterial processes shown to depend on quorum sensing [4–7], and are modulated by light [14]. As has been extensively described, our results also show that AbaI is involved in these responses. Interestingly, complementation of the *ΔabaI* mutant with *abaI* expressed from a plasmid restored photoregulation of motility at moderate temperatures suggesting that AbaI is a direct or indirect component of the light signaling cascade. The production of AHL was found to be modulated by light on cells recovered both from motility and biofilm assays at moderate temperatures, as determined by the use of the *C. subtsugae* CV026 and *C. violaceum* VIR07 biosensors, in a BlsA and AbaI-dependent manner. In fact, AHL production followed motility and biofilm formation patterns in the different strains. In agreement, *abaI* expression was found to be stimulated in the dark, which followed AHL production. Surprisingly, despite significant and similar levels of AHLs were produced in the *blsA* mutant under blue light and in the dark, expression of *abaI* was low and similar to the wild type under blue light in this mutant, showing no correlation between *abaI* expression, motility and production of AHLs at environmental temperatures. A possible explanation for these results could be that BlsA modulates *abaI* expression "timing". Then, *abaI* expression could have been significant in the *blsA* mutant both under blue light and in the dark in a time lapse previous to sample recovery, allowing thus AHLs production and motility, and could have decreased reaching minimal expression latter, which is a typical trait of the AHL expression curve. Quorum quenching activity was found to be higher under blue light than in the dark at environmental temperatures, in a BlsA dependent-manner. Consistently, expression patterns of *aidA*, reported to function as a lactonase [26], are also in agreement with the AHL pattern under blue light and darkness. In particular, *aidA* expression was found to be induced under blue light at environmental temperatures, which is consistent with the null or reduced presence of AHL in this condition and increased quorum quenching activity detected in this condition. At temperatures compatible with warm-blooded hosts such as 37ºC, modulation of the quorum network by light was also observed. However, the pattern was opposite to that observed at environmental temperatures. In fact, AHL production was higher under blue light than in the dark in a BlsA-independent AbaI-dependent manner. In addition, *aidA* expression was found to be induced in the dark, which is consistent with less AHL production and higher quorum quenching activity at this condition. It should be noted that the net amount of AHLs at each condition is the result of the contribution of the activities of AbaI, AidA as well as other putative lactonases or quorum quenching molecules not yet described or studied in the present work. Overall, we show that there is a fine tuning of quorum sensing vs. quenching activities dictated by the influence of light on expression of AHLs synthases and lactonases and integrating also a temperature signal, which results in differential production of AHLs in response to illumination and temperature.

Figure 8 summarizes the working model depicting light modulation of the *A. baumannii* QN based on results obtained in this work [11]. At environmental temperatures such as 23ºC, the photoreceptor BlsA interacts with AbaR only in the dark most probably with the bound AHL, inducing expression of the *abaI* gene and the production of AHL at this condition (Fig. 8A). At environmental temperatures such as 23ºC but in the presence of light, BlsA does not interact with AbaR, most likely because it is in a non-permissive conformation (Fig. 8B). Interestingly, BlsA induces of expression of the AidA, reported as a lactonase, in the in the presence of light but not in the dark. This is consistent with the increased quorum quenching activity observed in the presence of light, which is even more deepened in the *ΔabaI* mutant. The overall result is the presence of higher levels of AHL in the dark than under blue light, which correlates with higher motility (Fig. 8A and B).

At 37ºC, BlsA does not interact with AbaR, neither in the dark, nor under blue light, indicating that this photorreceptor is not involved in the quorum response at this temperature. *abaI* expression levels, as well as AHLs are higher in the presence of light than in the dark. Interestingly, *aidA* expression levels were induced in the dark and significantly increased in the *abaI* mutant both in the dark and under blue light, indicating that AbaI or its products inhibit expression of the *aidA* lactonase.

Moreover, we show that AbaI contributes to modulation of *A. baumannii*'s virulence by light, reducing *C. albicans* killing in the dark respect to light conditions both at environmental temperatures as well as temperatures found in warm-blooded hosts. These results are in agreement with others' indicating that the mutation of the AbaI synthase significantly reduces virulence in *in vivo* models [28,29].

It has been reported that N-hydroxydodecanoyl-L-homoserine lactone (OHC12-HSL) is the main QS signal produced by the clinically relevant species belonging to the *A. calcoaceticus– A. baumannii* complex, including the well-studied strains *A. nosocomialis* M2 and *A. baumannii* ATCC 17978TM, although other AHLs are also produced in smaller amounts [5](Niu et al., 2008; Chan et al., 2011, 2014; Clemmer et al., 2011; How et al., 2015; Mayer et al., 2018). In addition, up to 8 putative QQ enzymes have been found in the genome of *A. baumannii* ATCC 17978TM [30]. Our results are in agreement with these reports, as we detect the presence of short as well as long chain AHL, as well as non-diffusible quorum quenching activity compatible with a lactonase/s. It should be noted that many of our experiments were performed at environmental temperatures, and therefore different sets of AHLs not previously described could be produced. The identification of the different AHLs produced under the different illumination conditions is an interesting task, however, beyond the scope of this work.

Integration of quorum and light signals to modulate collective behaviors in *P. aeruginosa* have been recently reported [31]. In particular, the response regulator AlgB has been shown to be the node that integrates three inputs: quorum sensing through the activating action of the quorum sensing receptor RhlR, light through the BphP photorecepor, and an unknown signal via its partner KinB, to modulate biofilm formation and virulence [31]. In this work, we show that light directly modulates the quorum network. Evidence of this includes the interaction of the photoreceptor BlsA with AbaR in the dark but not in the presence of blue light at environmental temperatures. Also, expression of *abaI* and *aidA* are

modulated by light, and finally the presence of AHL is differential depending on the illumination conditions. Thus, a new concept is introduced in this work with advances on mechanistical insights. However, further work is still required to completely elucidate the mechanism of light regulation of quorum sensing in *A. baumannii*, which surely involves many other effectors. For example, the role of AbaM in the model has yet to be established, as it may also participate directly in modulation of the response to light and likely interact with BlsA.

Methods

Bacterial Strains, Plasmids, and Media. Bacterial strains and plasmids used in this work are listed in Table 1. Luria-Bertani (LB) broth (Difco) and agar (Difco) were used to grow and maintain bacterial strains. Broth cultures were incubated at the indicated temperatures either statically or with shaking at 200 rpm.

Table 1
Bacterial, yeast strains and plasmids used in this study.

Strain/Plasmid	Relevant Characteristic	Source or Reference
A. baumannii		
ATCC 17978	Clinical isolate	ATCC
ATCC 17978 *ΔabaI*	Generated by mutagenesis using plasmid pMO130-telR	37
ATCC 17978 *ΔblsA*	*blsA*::aph derivative of 17978; Km^r	14
ATCC 17978 *ΔblsA* pWHBlsA	17978 *ΔblsA* harboring plasmid pWH*blsA*; Km^R Amp^R	14
ATCC 17978 *ΔblsA* pWH1266	17978 *ΔblsA* harboring pWH1266; Km^R Tet^R Amp^R	14
ATCC 17978 *ΔabaI* pWHAbaI	17978 *ΔabaI* harboring plasmid pWH*abaI*; Km^R Amp^R	This study
ATCC 17978 *ΔabaI* pWH1266	17979 *ΔabaI* harboring plasmid pWH1266; Km^R Tet^R Amp^R	This study
Chromobacterium subtsugae		
CV026	AHLs short chain biosensor, *cvi*I::mini-Tn5 KnR	25 35
Chromobacterium violaceum		
VIR07	AHLs long chain biosensor *cvi*I mutant	36
E. coli		
DH5α	Used for DNA recombinant methods	Gibco-BRL
Saccharomyces cerevisiae		
Mav 203 strain	MATa, *leu*2-3,112, *trp*1-901, *his*3-D200, *ade*2-101, *gal*4D, *gal*80D, SPAL10::URA3, GAL1::*lacZ*, HIS3UAS GAL1::HIS3, YS2, *can*1R and *cyh*2R	Thermofisher
Plasmids		
pBluescript	PCR cloning vector; Ampr	Promega

Strain/Plasmid	Relevant Characteristic	Source or Reference
pWH1266	*E. coli-A. baumannii* shuttle vector; Amp^R Tet^R	38
pWHBlsA	pWH1266 harboring wildtype copy of *blsA* from ATCC 17978 expressed under its own promoter; Amp^R	14
pWHAbaI	pWH1266 harboring wild-type copy of *abaI* from ATCC 17978 expressed under its own promoter; Amp^R	This study
pENTR3C	Gateway system entry-vector	Invitrogen-Thermofisher
PGAD-T7-GW	Y2H AD-fusion vector, adapted to Gateway System	Clontech, 32
PGBK-T7-GW	Y2H DB-fusion vector, adapted to Gateway System	Clontech, 32

Plasmid Construction

Y2H. PCR amplifications of *blsA* and *acoR* coding sequences were performed from *A. baumannii* ATCC 17978 genomic DNA using primers *blsAdh* [21] and *abaRdh* (Table 2). The amplification products were subsequently cloned into the BamHI and XhoI sites of Gateway entry vector pENTR3C (Invitrogen) (Tabla 1). The cloned fragments were then transferred to pGBKT7-Gw and pGADT7-Gw Y2H vectors (Clontech) by using LR Clonase [20,21,32]. In the yeast host, these plasmids express the cloned coding sequences as fusion proteins to the GAL4 DNA-binding domain (DB) or activation domain (AD), respectively, under the control of the constitutive ADH1 promoter. Automated DNA sequencing confirmed correct construction of each plasmid.

Table 2
Primers used in this study.

Name	SEQUENCE (5'-3')	REFERENCE
abaRdhF	GGATCCATGGAAAGTTGGCAAGAAGATTT	This study
abaRdhR	CTCGAGACCTACAAAAGCCCTAGCATTACAG	This study
blsAdhF	GGATCCATGAACGTTCGCCTGTGT	20
blsAdhR	CTCGAGTGCTAGAACGGGTTTACTC	20
pabaIF	GGATCCTACAAGTGCTTCCACTTATTTTTCA	This study
pabaIR	GGATCCTTTCTTATATAGGACTCATGCCT	This study
aidAF	GGGAACTTCTTTCGGTGGAG	This study
aidAR	AACAGCAGCAAGTCGATTATCA	This study
abaIF	CCGCTACAGGGTATTTGTTGAAT	This study
abaIR	GCAGGGAATAGGCATTCCATTG	This study
rpoBF	CAGAAGTCACGCGAAGTTGAAGGT	17
rpoBR	AACAGCACGCTCAACACGAACT	17
recAF	TACAGAAAGCTGGTGCATGG	14
recAR	TGCACCATTTGTGCCTGTAG	14

pWHAbaI. *abaI* coding sequence and its promoter region were amplified by PCR using *A. baumannii* ATCC 17978 genomic DNA as template and primers p*abaI*F and p*abaI*R (Table 2), which contained BamHI restriction site tails. The amplification product was cloned into pWH1266 through the BamHI site, following protocols described in Mussi *et al.*, 2010. Automated DNA sequencing confirmed the proper construction of pWHAbaI plasmid.

Blue Light Treatments. Blue light treatments were conducted as reported before [14,15,17, 20–22,33]. Briefly, cells were grown in the dark or under blue light emitted by an array composed of 3x3-LED module strips emitting an intensity of 6–10 mol photons/m^2/s, with emission peaks centered at 462 nm [14].

Yeast Two-Hybrid (Y2H) Assays. Yeast two-hybrid experiments were conducted following procedures described before [20,21,32]. *Saccharomyces cerevisiae* Mav 203 strain (MATα, *leu*2-3,112, *trp*1-901, *his*3-Δ200, *ade*2-101, *gal*4Δ, *gal*80Δ, SPAL10::URA3, GAL1::*lac*Z, HIS3UAS GAL1::HIS3, LYS2, *can*1R, and *cyh*2R) was transformed with the different expression vectors. First, BlsA and AbaR were analyzed for self-activation. For this purpose, MaV203 yeast strain containing the pGAD-T7 empty vector was

transformed with the DNA DB-fusion protein expressing vectors (pGBK-X) (X = BlsA or AbaR). Conversely, MaV203 yeast strain containing the pGBK-T7 empty vector was then transformed with the AD-fusion protein expressing vectors (pGAD-Y) (Y = BlsA or AbaR). In addition, these strains were used for determination of the optimal 3-amino-1,2,4-triazole (3AT) concentration required to titrate basal HIS3 expression. MaV203/pGBK-X strains were afterward transformed with each pGAD-Y plasmids. Transformations using one or both Y2H plasmids were performed by the lithium acetate/single-stranded carrier DNA/polyethylene glycol method described in Gietz and Woods (2002), and plated in convenient minimal selective medium [synthetic complete (SC) medium without leucine (-leu) for pGAD-Y transformants, SC without tryptophan (-trp) for pGBK-X transformants, and SC-leu-trp transformants carrying both plasmids]. The plates were then incubated at 30ºC for 72 h to allow growth of transformants. A “Master Plate” was then prepared using SC-leu-trp media, in which we patched: four to six clones of each pGBK-X/pGAD-Y containing yeasts, four to six self-activation control clones pGBK-X/pGAD and pGBK/pGAD-Y (Y DNA-binding negative control), and two isolated colonies of each of the five yeast control strains (A–E). The plates were incubated for 48–72 h at 23 ºC in the dark. This Master Plate was then replica plated to SC–leu–trp–hisC 3AT and to SC–leu–trp–ura to test for growth in the absence of histidine (his) and uracil (ura), respectively (*his3* and *ura3* reporter activation), under the different conditions analyzed, i.e., dark/light; 23/30 ºC, for at least 72 h. For development of blue color as a result of β -galactosidase (β-Gal) expression, transformed yeasts were replica plated on a nitrocellulose filter on top of a YPAD medium plate and grown at the different conditions (dark/light; 23/30 ºC). Then, the cells on the nitrocellulose filter were permeabilized with liquid nitrogen and soaked in X-Gal solution (5-bromo- 4-chloro-3-indolyl-b-D-galactopyranoside in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0), for 24 hours at 37ºC following the manufacturer's recommendations (Invitrogen).

Analyses of gene expression by qRT-PCR. Retrotranscription and qRT-PCR analysis were done as described in Tuttobene et al., 2019, using primers listed in Table 2. Data are presented as NRQ (Normalized relative quantities) calculated by the qBASE method [34], using *recA* and *rpoB* genes as normalizers.

Cell motility assay. Cell motility was tested on swimming agarose media: 1% tryptone, 0.5% NaCl and 0.3% agarose plates inoculated on the surface by depositing 3µl of LB cultures grown to an optical density at 660nm (OD_{660}) of 0.3. The plates were incubated 24 or 48 hours in the presence or absence of blue light at 37°C or 23ºC, respectively. For AHL detection assays, the aqueous media containing the cells on the surface of motility plates was recovered, homogenized, and the optical density at 660nm was determined. Cultures were then centrifuged, filtered-sterilized and amounts normalized to OD_{660} = 1.5 were loaded on biosensor plates, as is indicated in section AHLs detection using biosensors. Triplicate assays were done using fresh samples each time.

Biofilm formation assays. For biofilm assays, two milliliters of fresh swimming agarose broth medium contained into glass tubes were inoculated with 0.01 ml of an overnight shaking culture grown at 37°C.

The cultures were then incubated stagnantly at 23°C or 37ºC either in darkness or under blue light for 48 or 24 hs, respectively. Biofilms and pellicles that formed on the walls of the glass tubes were detected by visual inspection [14]. Replicate tubes were homogenized by vortexing and the cell density was determined by measurement of OD_{660}. In general, no significant differences were registered between the different strains incubated under blue light vs. dark conditions, at each temperature. Nonetheless, whether any difference in optical density appeared, the amount of supernatants was normalized to OD_{660} = 0.5. Each culture was then centrifuged, and the supernatants were filter-sterilized and used for AHL detection, as indicated in the following item. Triplicate assays were done using fresh samples each time.

AHLs detection using biosensors. Different biosensors, namely *Chromobacterium subtsugae* (formerly *C. violaceum*) ATCC 31532 CV026 [35] and *Chromobacterium violaceum* ATCC 12472 VIR07 [36], were used to detect the presence of AHL in *A. baumannii* cultures recovered from motility plates or biofilm tubes. *Chromobacterium* contains the CviI/R AHL QS system. *C. subtsugae cviI* mutant CV026 [25] does not produce AHL, but induces the CviR upon exposure to exogenous short-chain AHLs, which results in rapid synthesis of a visually clear purple pigmentation, violacein. Particularly, violacein is inducible by compounds with N-acyl side chains from C_4, to C_8, in length, with varying degrees of sensitivity [25]. C6-AHL induces maximum pigment production. Other AHLs which induce the CviR relatively well include C6-3-oxo-AHL, C8-AHL, C8-3-oxo-AHL and C4-AHL. The CV026 biosensor is least responsive to C4-3-oxo-AHL and AHLs with acyl chains of C10 and longer. Although AHL compounds with N-acyl side chains from C_{10} to C_{14} are unable to induce violacein production, if an activating AHL is incorporated into the agar, these long-chain AHLs can be detected by their ability to inhibit violacein production [25]. In turn, violacein production in *C. violaceum* ATCC 12472 *cviI* mutant VIR07 is induced in response to the long-chain AHLs (C10–C16), and the most active AHL in this assay was C10-HSL [36]. In contrast, there was no or a very weak (data not shown) response to the short-chain AHLs (C4–C8). Violacein production was inhibited by short-chain AHLs (C6–C8), but not by C4-HSL.

Supernatants recovered from motility plates or biofilm tubes (approximately 500µl), were loaded in a central well of LB plates previously inoculated with *C. subtsugae* CV026 or *C. violaceum* VIR07. *Chromobacterium* inoculation was performed by addition of 500 µL of overnight cultures grown to an OD_{660} = 2.5 to 5 ml of 0.7% agar media, which was then incorporated on top of the 1.5 % agar LB plates. The plates were then incubated at 30ºC in the dark for 24 h.

Quantification of violacein production in the different strains was determined by measuring the area and integrated density of each complete plate and subtracting the corresponding values measured in the negative control, using ImageJ software (NIH). The data were then normalized respect to the positive control, which received the arbitrary value of 100. The data shown are the means of three independent experiments, and error bars represent the standard deviation of the mean.

AHL quorum quenching activity assay. Cells recovered from motility plates of the indicated strains incubated under blue light or in the dark at 23ºC or 37ºC for 48 or 24 hours respectively, were normalized

to OD_{660} = 1.5. The cells were recovered by centrifugation at 3000 g for 10 min and resuspended in 500 µL in swimming media (1% tryptone, 0.5% NaCl). The cells were sonicated and then centrifuged. Post-sonication supernatants were incubated with 2 µg of commercial standards, C8-AHL or C10-AHL, for 6 hours in a shaker at 37ºC. Aliquots (500 µl) of the resulting supernatant were used to detect AHL degradation in a well diffusion assay in double agar plates, in which *C. subtsugae* CV026 or *C. violaceum* VIR07 were added to soft agar as a biosensors to detect inhibition of violacein [26]. Quantification of violacein production in the different strains was determined by measuring the area and integrated density of each complete plate and subtracting the corresponding values measured in the negative control, using ImageJ software (NIH). The values were normalized to the positive control, which received the arbitrary value of 100. The data shown are the means of three independent experiments, and error bars represent the standard deviation of the mean.

Killing of *C. albicans* filaments. Assays were performed as described before [14,22], with the modification of incubating 1-ml of the co-cultures without shaking at 37°C from 24 hours to 72 hours under dark or blue light conditions. Fungal CFU counts per ml were determined at each time point studied by plating convenient dilutions of the co-cultures on yeast extract-peptone-dextrose (YPD) agar containing 60mg/ml tetracycline, 30mg/ml chloramphenicol, and 30mg/ml gentamicin, following incubation at 28°C for 48 hours.

Statistical analysis. Experiments were performed in technical and biological triplicates and ANOVA followed by Tukey's multiple-comparison test ($P < 0.05$) statistical analyses were performed using GraphPad Prism (GraphPad software, San Diego, CA, USA).

Declarations

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Author contribution statement: MRT, GLM, LB, LD and PC performed experiments. CNP performed experiments and collaborated in writing the manuscript. MT provided material and collaborated in writing the manuscript. MAM designed experiments, wrote the manuscript and provided funding. All authors reviewed the manuscript.

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Figures

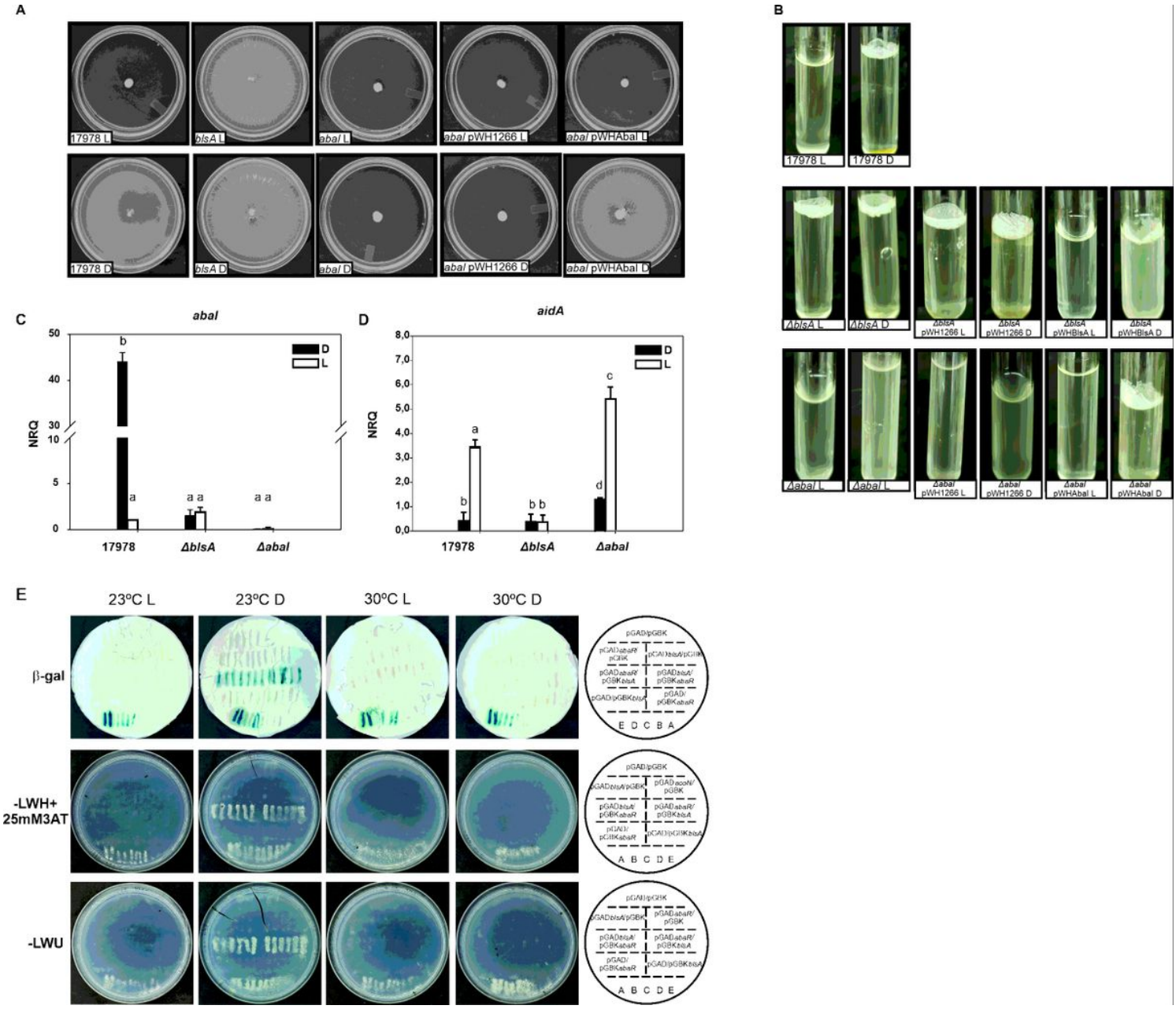

Figure 1

Contribution of AbaI to modulation by light of motility and biofilm formation in A. baumannii at environmental temperatures, and BlsA-AbaR interaction. (A) Cells of the parental strain ATCC 17978, the isogenic ΔblsA and ΔabaI mutants, as well as this mutant harboring the empty pWH1266 plasmid or the abaI-complementing plasmid pWHAbaI were inoculated on the surface of motility plates. Plates were inspected and photographed after incubation in darkness (D) or in the presence of blue light (L) at 23°C. Representative results of three independent experiments are shown. (B) Cells of the parental strain ATCC 17978, the isogenic ΔblsA and ΔabaI mutants, as well as these mutants harboring the empty pWH1266 plasmid, the abaI-complementing plasmid pWHAbaI or the blsA-complementing plasmid pWHBlsA were inoculated on swimming media-containing tubes and incubated stagnantly for 48 hs at 23ºC. Representative results of three independent experiments are shown. (C-D) abaI and aidA expression is modulated by light in A. baumannii at environmental temperatures. Estimation by qRT-PCR of the

expression levels of the gene coding for an acyl homoserine lactone synthase, abaI (C), and the gene encoding a lactonase, aidA (D), in cells recovered from motility plates inoculated with ATCC 17978 wild-type, ΔblsA and ΔabaI incubated at 23°C under blue light (L) or in the dark (D). Shown are the mean and standard deviation of normalized relative quantities (NRQ). Significant differences determined by ANOVA followed by Tukey's multiple comparison test ($p < 0.05$) are indicated by different letters. Shown are representative results of three independent experiments. (E) BlsA–AbaR interaction studied by Y2H assays. Six clones of MaV203/pGAD-blsA or MaV203/pGAD-abaR transformed with plasmids pGBK-abaR or pGBK-blsA, respectively, as well as plasmid pGBK-T7 or pGAD-T7 as negative control, were patched in each plate. Also included are reciprocal combinations, and self-activation as well as different strength interaction controls (strains A–E). The description and order of yeast streaks on each plate are indicated in the scheme on the right. Results for the lacZ reporter, the histidine auxotrophic marker and the uracil reporter are indicated in the top, middle and bottom panels. Experiments were performed in triplicate and representative results are shown.

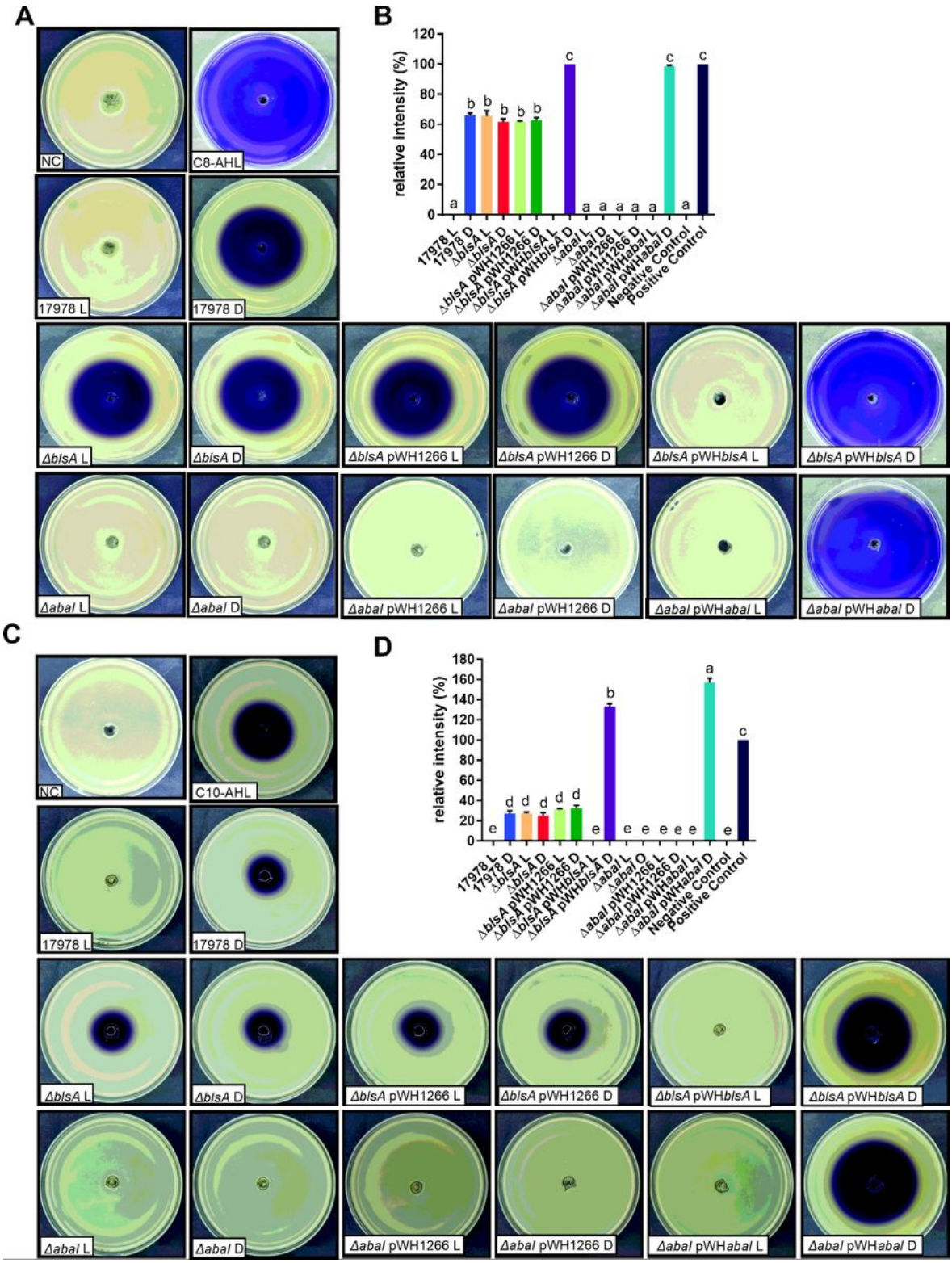

Figure 2

Light modulates AHLs production in motility in A. baumannii at environmental temperatures. Supernatants recovered from motility plates of the indicated strains incubated under blue light or in the dark at 23ºC were filtered sterilized, and amounts normalized to a OD660=1.5 were then loaded in a central well of biosensor-inoculated LB plates. The C. subtsugae CV026 biosensor (A) produces violacein as a result of the presence of short-chain AHLs in the supernatants (C4 to C8); while C. violaceum VIR07

(C) senses long-chain AHLs (C10-C16). The negative control (NC) was performed by inoculating PBS 1X in the central well of biosensor-inoculated LB plates. The positive controls (labeled C8-AHL or C10-AHL) were performed by loading 2 µg of commercial standard to the central well of the biosensor-inoculated LB plates. Plates were inspected and photographed after incubation in darkness (D) at 30°C for 24 hs. Representative results of three independent experiments are shown. (B-D) Quantification of violacein production in the different strains was determined by measuring the area and integrated density of each complete plate and subtracting the corresponding values measured in the negative control, using ImageJ software (NIH). The values were normalized to the positive control, which received the arbitrary value of 100. The data shown are the means of three independent experiments, and error bars represent the standard deviation of the mean. Different letters indicate significant differences as determined by ANOVA followed by Tukey's multiple comparison test ($p<0.05$).

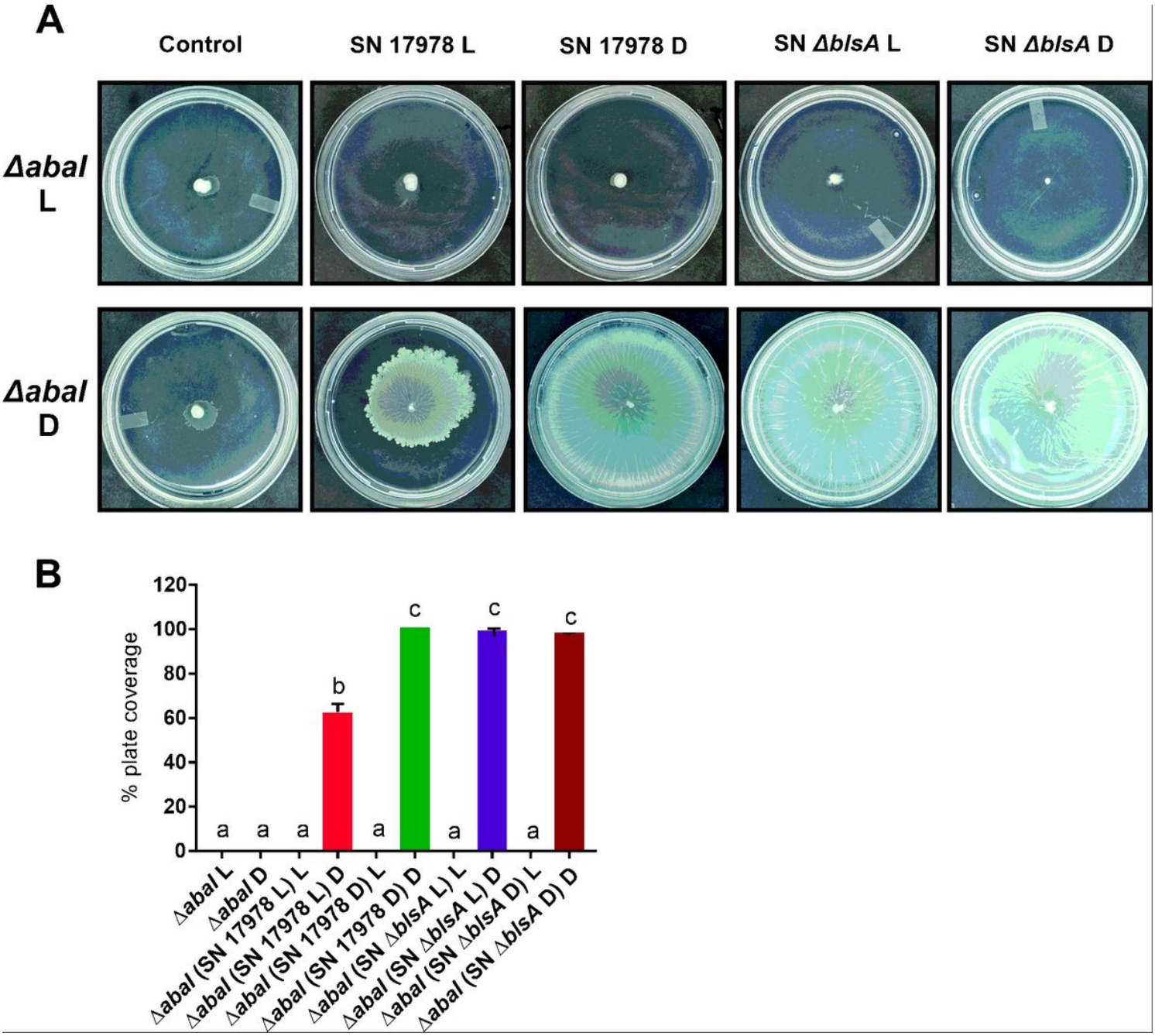

Figure 3

A. baumannii produces diffusible molecules able to restore the ΔabaI motility defect. (A) Supernatants (SN) recovered from motility plates of A. baumannii ATCC 17978 or the isogenic ΔblsA mutant incubated under blue light or in the dark at 23ºC were filtered sterilized, and amounts normalized to a OD660=1.5 were then used to supplement the swimming agarose media of plates inoculated with ΔabaI. The control was performed by supplementing the media with PBS 1X. Plates were inspected and photographed after incubation in darkness (D) or in the presence of blue light (L) at 23°C for 24 h. Representative results of three independent experiments are shown. (B) Quantification of cell motility estimated as the percentage of plate coverage, i.e., the percentage of the Petri plate area covered with bacteria, in motility plates supplemented with the different supernatants and inoculated with ΔabaI. The area of plates covered with bacteria was measured with ImageJ (NIH), and then the percentage of plate coverage was calculated. The mean and standard deviation of three independent experiments are informed. Significant differences determined by ANOVA followed by Tukey's multiple comparison test ($p < 0.05$) are indicated by different letters. For those conditions at which the bacteria just reached the edge of the plate a value of 100% is informed.

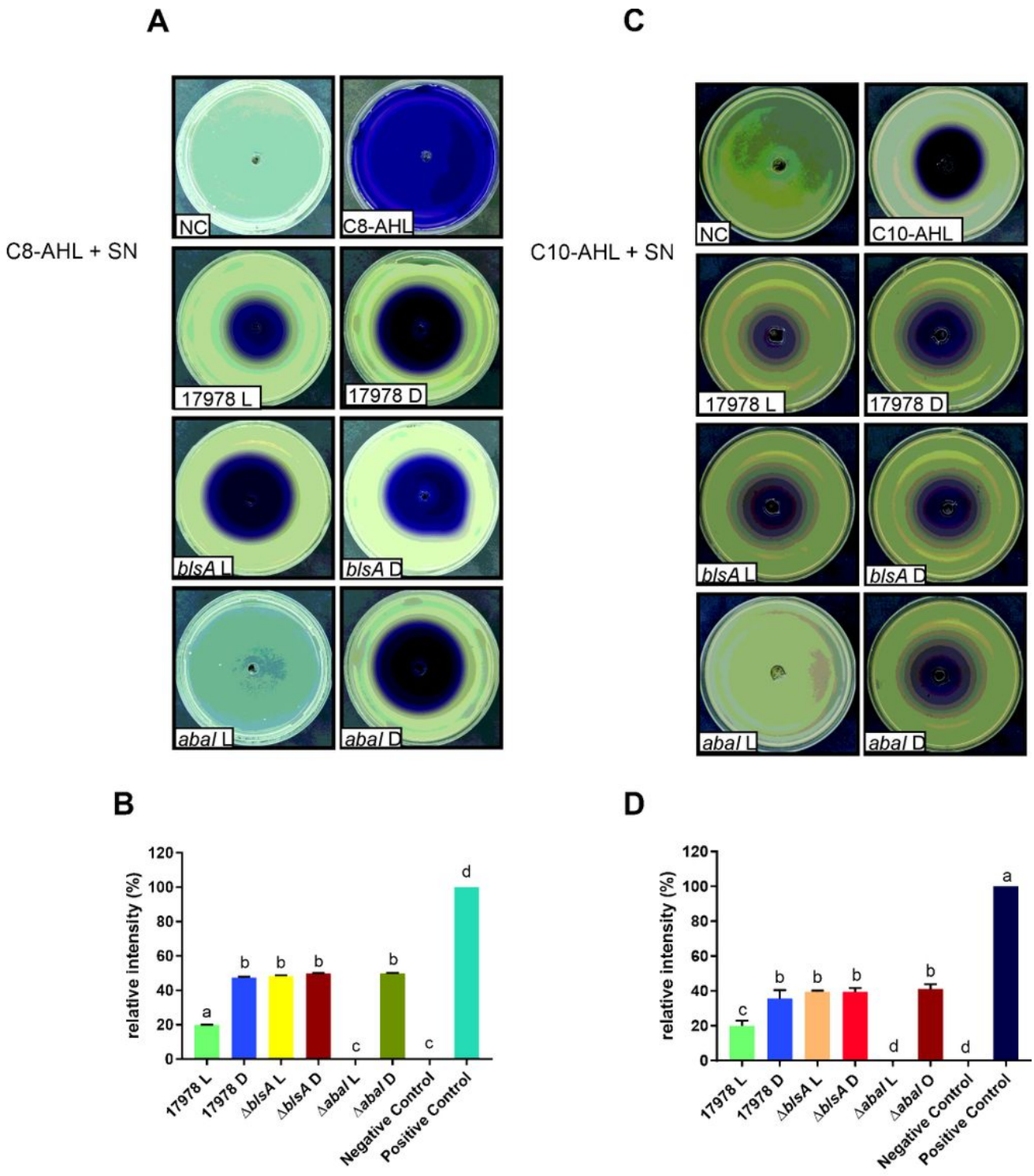

Figure 4

Light modulates quorum quenching activity at environmental temperatures in A. baumannii. Quorum quenching assays were performed by incubating the post-sonication supernatants recovered from motility plates of the indicated strains incubated under blue light or in the dark at 23ºC and normalized to OD660=1.5, with 2 µg of commercial standard for 6 hours at 37ºC. After incubation, the mixture was then loaded in a central well of biosensor-inoculated LB plates. The biosensors used are the short-chain AHL

biosensor C. subtsugae CV026 (A) as well as the long-chain AHL biosensor C. violaceum VIR07. (C) Negative control (NC) was performed by inoculating PBS 1X in the central well of the plates. The positive controls (standards labeled C8-AHL or C10-AHL) were performed by adding 2 µg of each commercial standard. The extent of inhibition of violacein production compared to the control provides an indication of lactonase activity. Plates were inspected and photographed after incubation in darkness (D) at 30°C for 24 h. Shown are representative results from three independent experiments. (B-D) Quantification of violacein production in the different strains was determined by measuring the area and integrated density of each complete plate and subtracting the corresponding values measured in the negative control, using ImageJ software (NIH). The values were normalized to the positive control, which received the arbitrary value of 100. The data shown are the means of three independent experiments, and error bars represent the standard deviation of the mean. Different letters indicate significant differences as determined by ANOVA followed by Tukey's multiple comparison test ($p<0.05$).

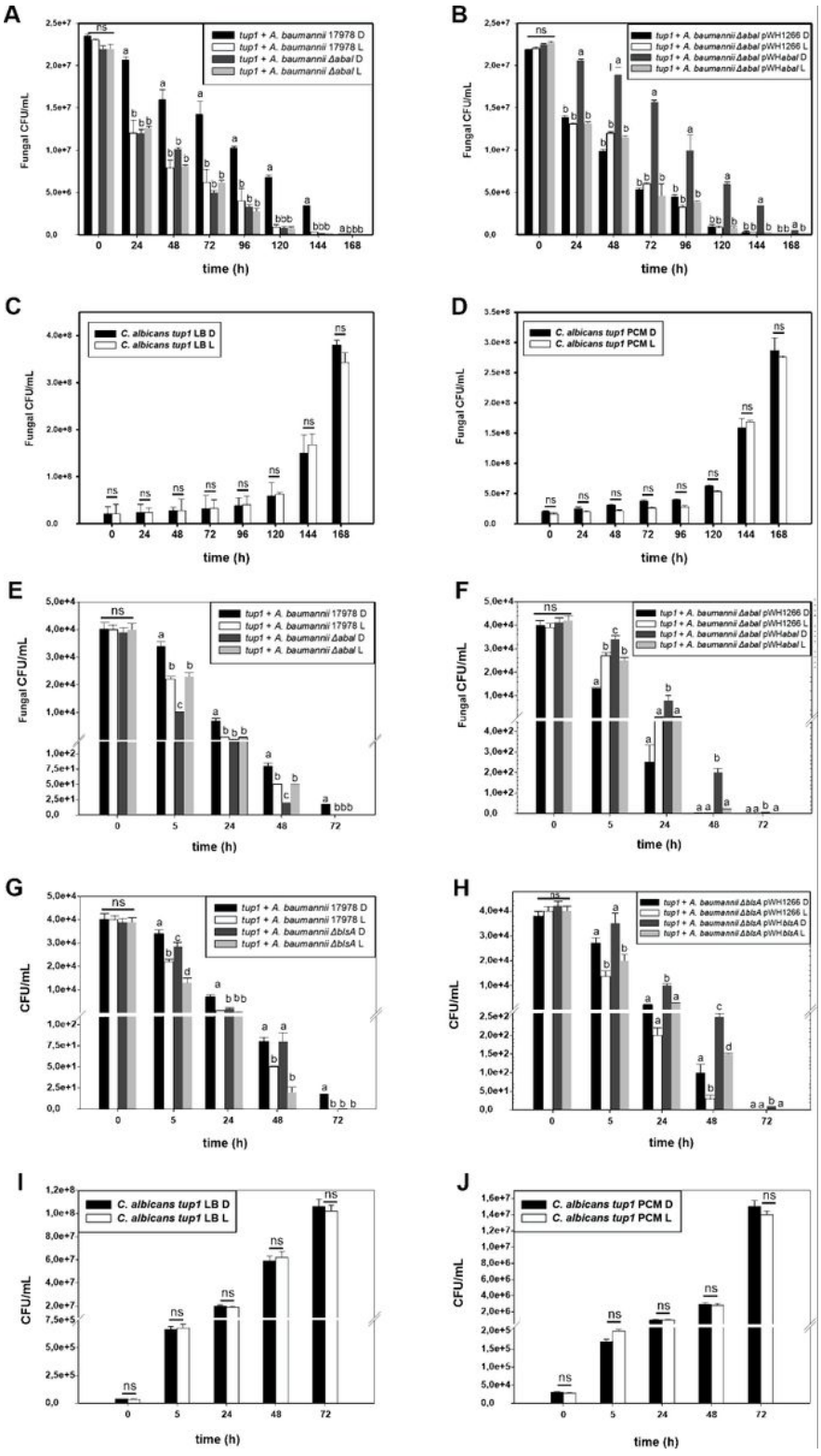

Figure 5

Killing of tup1 mutant C. albicans by A. baumannii cells in response to light. (A-D) Killing of tup1 mutant C. albicans by A. baumannii cells in response to light at environmental temperatures. (A) Fungal filaments were co-incubated with bacterial cells from the ATCC 17978 parental strain or the isogenic ΔabaI mutants in presence of the corresponding filtered sterilized preconditioned medium under blue light (L) or in darkness (D) at 23°C. (B) Similar to experiment shown in panel A but using the mutant

harboring the empty pWH1266 plasmid or the complementing plasmid pWHAbaI. Aliquots were taken at different times and plated on selective media to counter select for A. baumannii and follow C. albicans survival (see material and methods for details). Shown are the means and standard deviations from three replicates. Different letters indicate significant differences as determined by ANOVA followed by Tukey's multiple comparison test ($p < 0.05$). Representative results from three independent experiments are shown in each case. (C and D) Controls performed by co-incubation of C. albicans alone in the presence of LB or PCM, respectively. (E-J) Killing of tup1 mutant C. albicans by A. baumannii cells in response to light at 37ºC. Fungal filaments were co-incubated with bacterial cells from the ATCC 17978 parental strain and the ΔabaI mutant (E) or the ΔblsA mutant (G) in presence of the corresponding filtered sterilized preconditioned medium under blue light (L) or in darkness (D) at 37ºC. (F and H) Similar to experiment shown in panel E and G but using the mutants harboring the empty pWH1266 plasmid or the complementing plasmids pWHAbaI or pWHBlsA, respectively. Aliquots were taken at different times and plated on selective media to counterselect for A. baumannii and follow C. albicans survival (see material and methods for details). Shown are the means and standard deviations from three replicates. Different letters indicate significant differences as determined by ANOVA followed by Tukey's multiple comparison test ($p < 0.05$). Representative results from three independent experiments are shown in each case. (I and J) Controls performed by co-incubation of C. albicans alone in the presence of LB or PCM, respectively.

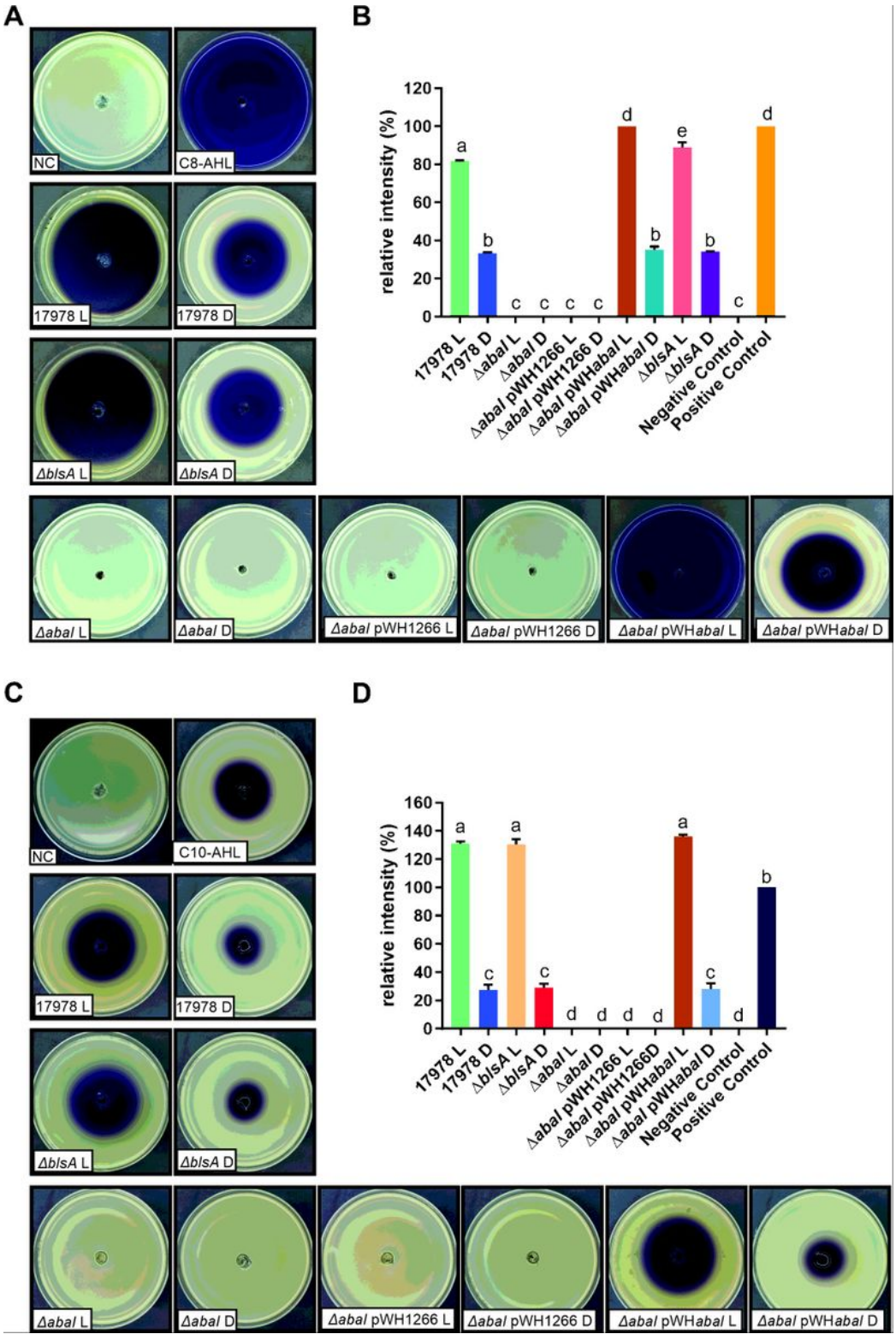

Figure 6

Light modulates AHLs production in motility in A. baumannii at 37ºC. Supernatants recovered from motility plates of the indicated strains incubated under blue light or in the dark at 37ºC were filtered sterilized, and amounts normalized to a OD660=1.5 were then loaded in a central well of biosensor-inoculated LB plates. The C. subtsugae CV026 biosensor (A) produces violacein as a result of the presence of short-chain AHLs in the supernatants (C4 to C8); while C. violaceum VIR07 (C) senses long-

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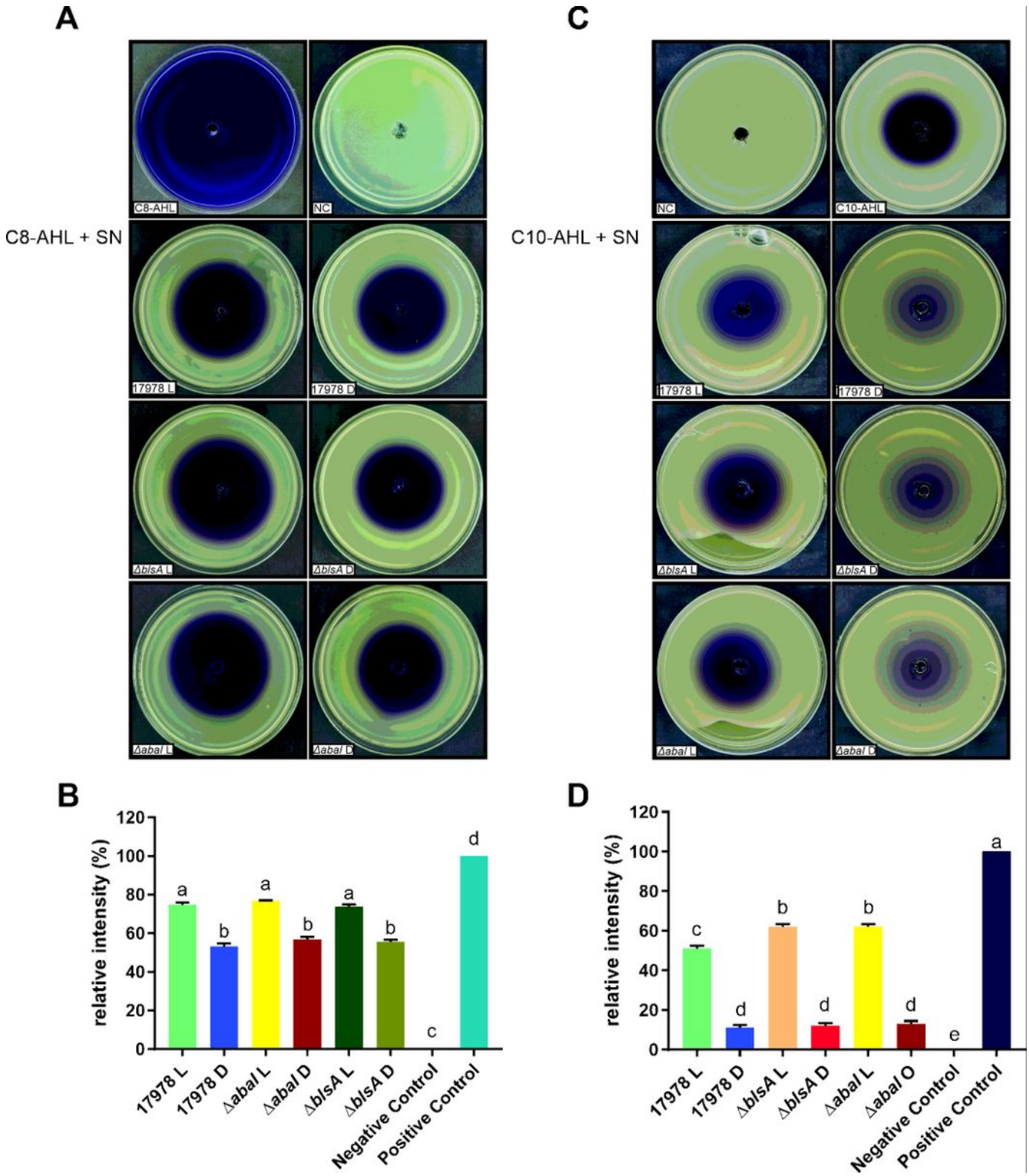

Figure 7

Light modulates quorum quenching activity in A. baumannii at 37ºC. Quorum quenching assays were performed by incubating the supernatants recovered from motility plates of the indicated strains incubated under blue light or in the dark at 37ºC and normalized to OD660=1.5, with 2 µg of commercial standard for 6 hours at 37ºC. After incubation, the mixture was then loaded in a central well of biosensor-inoculated LB plates. The biosensors used are the short-chain AHL biosensor C. subtsugae CV026 (A) as

well as the long-chain AHL biosensor C. violaceum VIR07. (C) Negative control (NC) was performed by inoculating PBS 1X in the central well of the plates. The positive controls (standards labelled C8-AHL or C10-AHL) were performed by adding 2 µg of each commercial standard. The extent of inhibition of violacein production compared to the control provides an indication of lactonase activity. Plates were inspected and photographed after incubation in darkness (D) at 30°C for 24 h. Shown are representative results from three independent experiments. (B-D) Quantification of violacein production in the different strains was determined by measuring the area and integrated density of each complete plate and subtracting the corresponding values measured in the negative control, using ImageJ software (NIH). The values were normalized to the positive control, which received the arbitrary value of 100. The data shown are the means of three independent experiments, and error bars represent the standard deviation of the mean. Different letters indicate significant differences as determined by ANOVA followed by Tukey's multiple comparison test ($p<0.05$).

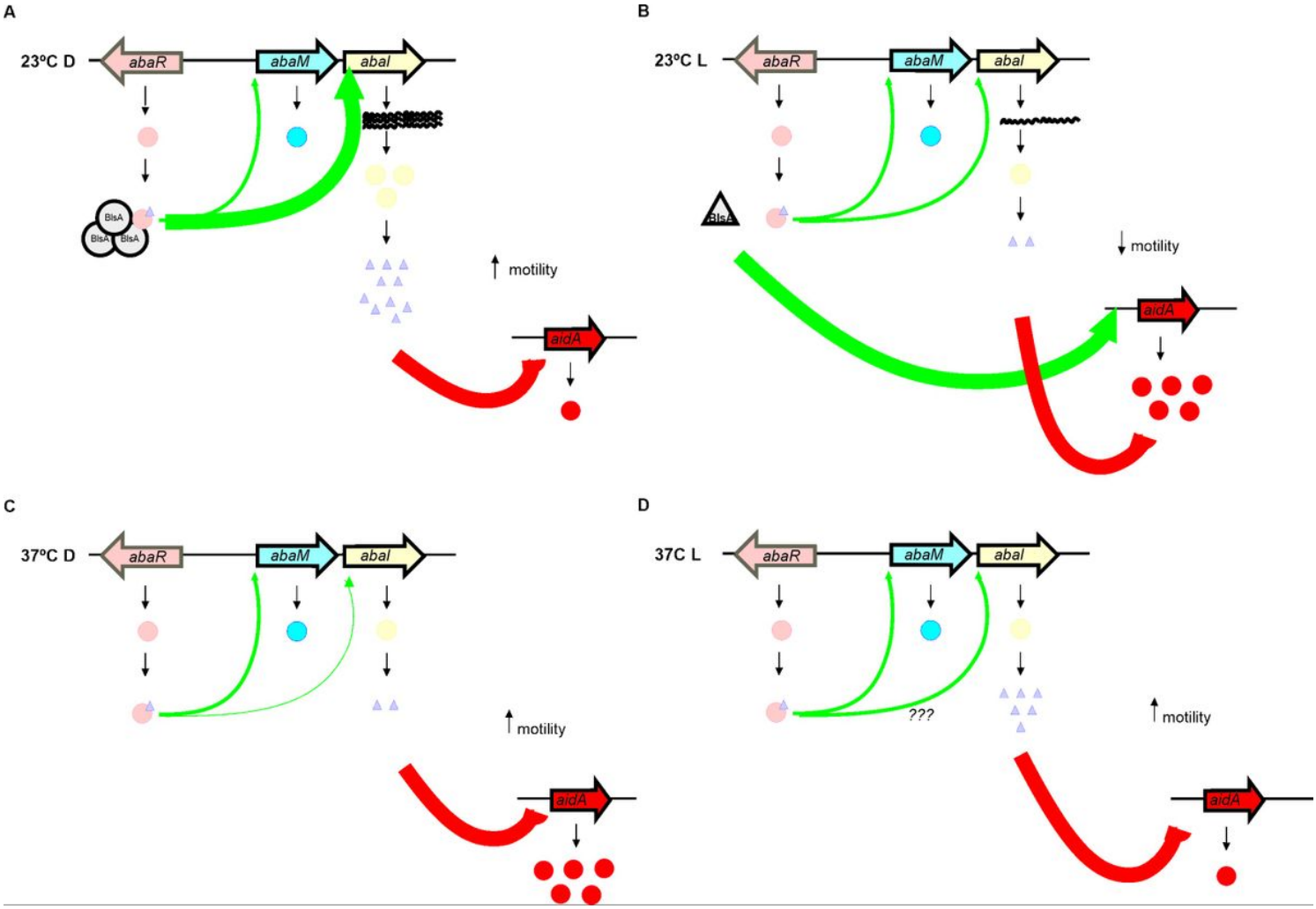

Figure 8

Working model depicting current knowledge regarding modulation by light of quorum sensing in A. baumannii. At environmental temperatures such as 23ºC, the photoreceptor BlsA interacts with AbaR only in the dark most probably with the bound AHL, inducing expression of the abaI gene and the production of AHL at this condition. At environmental temperatures such as 23ºC but in the presence of light, BlsA

does not interact with AbaR, most likely because it is in a non-permissive conformation. Interestingly, BlsA induces of expression of the AidA, reported as a lactonase, in the in the presence of light but not in the dark. This is consistent with the increased quorum quenching activity observed in the presence of light, which is even more deepened in the ΔabaI mutant. The overall result is the presence of higher levels of AHL in the dark than under blue light (Figure 8A and B). At 30ºC, and therefore we infer that neither does it at 37ºC, BlsA does not interact with AbaR, neither in the dark, nor under blue light (Figure 8C and D), indicating that this photorreceptor is not involved in the quorum response at this temperature. abaI expression levels, as well as AHLs are higher in the presence of light than in the dark. Interestingly, aidA expression levels were induced in the dark and significantly increased in the abaI mutant both in the dark and under blue light indicating that AbaI or its products inhibit expression of the aidA lactonase. However, this was not reflected in a higher quorum quenching activity in the abaI mutant.

Supplementary Files

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