Antimicrobial capacity of the freshwater planarians against \textit{S. aureus} is under the control of Timeless

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\textbf{ABSTRACT}

Planarians, which are non-parasitic flatworms, are highly resistant to bacterial infections. To better understand the mechanisms underlying this resistance, we investigated the role of the circadian machinery in the anti-bacterial response of the freshwater planarian \textit{Schmidtea mediterranea}. We identified \textit{Smed-Tim} from \textit{S. mediterranea} as a homolog of the mammalian clock gene \textit{Tim}. We showed via RNA interference that \textit{Smed-Tim} is required for the anti-microbial activities of \textit{Schmidtea mediterranea} against \textit{Staphylococcus aureus} infection during the light/dark cycle. Indeed, \textit{S. aureus} infection leads to the expression of \textit{Smed-Tim}, which in turn promotes \textit{Smed-Traf6} and \textit{Smed-morn2}, but not \textit{Smed-p38 MAPK} expression, 2 master regulators of planarian anti-microbial responses.

\textbf{KEYWORDS}

anti-bacterial response; MORN2; planarians; \textit{S. aureus}; \textit{Tim}

\textbf{Introduction}

Non-vertebrates such as fruit flies and nematodes have provided insight into the conserved mechanisms in host-pathogen interactions. The immortal \textit{Platyhelminthes planaria}, which is known for its ability to regenerate any part of its body,\textsuperscript{1} can be a useful tool to identify and characterize evolutionarily conserved anti-bacterial mechanisms due to its ability to eliminate a large spectrum of human pathogens.\textsuperscript{2} However, the mechanisms that govern planarian immunity remain largely unknown and require investigation.

To better understand the anti-microbial capacity of freshwater planarians, we investigated the contribution of the genes from the internal time keeping system, the circadian clock, to this process. Planarians, similar to numerous other organisms, have a circadian clock.\textsuperscript{3,4} It has been suggested that genes from the circadian cycle might play a role in the modulation of antibacterial immunity of non-vertebrates and vertebrates.\textsuperscript{5,7} Indeed, mice with Aryl Hydrocarbon Receptor Nuclear Translocator-Like (\textit{Arntl})-1 knockdown are more susceptible to \textit{Listeria monocytogenes} infection\textsuperscript{8} than wild type mice are. In non-vertebrates such as \textit{Drosophila melanogaster}, deletion of the clock gene \textit{Period}-2 (\textit{Per})-2 causes an increase in susceptibility to \textit{Pseudomonas aeruginosa}, \textit{Streptococcus pneumonia}, and \textit{L. monocytogenes} infections, whereas deletion of the clock genes \textit{Circadian Locomotor Output Cycles Kaput} (\textit{Clock}), Cycle (\textit{Cyc}), a homolog of \textit{Hs-Arntl-1}, and, although discussed, \textit{Timeless} (\textit{Tim}) could cause an increase in resistance to \textit{Pseudomonas aeruginosa}.\textsuperscript{6,9}

Based on these findings, we sought to determine the role of the circadian clock genes \textit{Arntl-1}, \textit{Tim}, \textit{Per-2}, and \textit{Clock} in the ability of the freshwater planarian \textit{Schmidtea mediterranea} to cope with bacterial infections. In this report, we identified \textit{Schmidtea mediterranea} (\textit{Smed})-\textit{TIM}, the planarian homolog of \textit{Homo sapiens} (\textit{Hs})-\textit{TIM}, as a component of the Clock machinery of planarians, required for anti-microbial activities involving \textit{Smed-traf6} and \textit{Smed-morn2} in the planarian against \textit{Staphylococcus aureus} during the light/dark cycle but not during the D/D cycle.

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\textsuperscript{b}Supplemental data for this article can be accessed on the publisher’s website.
Results

First, we have searched for homologs of the *Homo sapiens* (Hs)-Arntl-1, Hs-Tim, Hs-Per-2, and Hs-Clock genes in the *S. mediterranea* genome using the *S. mediterranea* transcriptome database PlanMine (http://planmine.mpi-cbg.de/planmine/begin.do)\(^1\) and the *S. mediterranea* genome database SmedGD (http://smedgd.neuro.utah.edu/).\(^1\) We were unable to find sequences with significant homologies to *Hs-Clock* and *Hs-Per-2* in either database, which suggests that *Clock* and *Per-2* are absent from *S. mediterranea*. In contrast, TBLASTN analysis provided 3 sequences (lcl|dd_Smed_v6_9171_0_1, e-value 4e^-77; lcl|dd_Smed_v6_17731_0_1, e-value 4e^-28; lcl|dd_Smed_v6_17537_0_1, e-value 4e^-22) with significant alignment to Hs-ARNTL-1 (NP_001025443.1) and 2 sequences (lcl|dd_Smed_v6_19257_0_2, e-value 2e^-82; lcl|dd_Smed_v6_17957_0_1, e-value 9e^-21) with significant alignment to Hs-TIM (NP_003911.2). Using FGENESH\(^2\) (http://www.softberry.com/), we predicted the Schmidtea mediterranea gene Smed-Arntl-1 as being homologous to *Hs-Arntl-1* and Smed-Tim as being homologous to *Hs-Tim*. Sequence conservation analysis with BLASTx and PRALINE\(^\text{\textsuperscript{12}}\) showed a 52% similarity at the protein level (68% coverage, e-value 1e^-142) for the predicted Smed-ARNTL-1 and Hs-ARNTL-1 (Fig. 1A) and a 31% similarity at the protein level (99% coverage, e-value 4e^-72) for the predicted Smed-TIM and Hs-TIM (Fig. 1B). The use of nLS Mapper software (http://nls-mapper.iab.keio.ac.jp/) identify the presence of a nuclear localization sequence (SRTKRRKSI) at the position 519, in the amino acid sequence of Smed-TIM (Fig. S1).

Second, we have investigated if Smed-Tim and Smed-Arntl-1 played a role in the anti-microbial response of the planarian *S. mediterranea*. Animals were subjected to either a light/dark (L/D) cycle (12/12 hours) or to a dark/dark (D/D) cycle (12/12 hours) and were then genetically deprived of Smed-Arntl-1 and Smed-Tim function via RNA interference. Planarians were then exposed to *S. aureus* by feeding. By the direct measurement of colony-forming units (CFUs), we evaluated bacterial clearance at 3, 6, and 9 d post-feeding. We observed that animals silenced for Smed-Arntl-1, or Smed-Tim (Fig. S2A) did not show any particular phenotype (Fig. 2A), and their viability was not affected (Fig. S2B). To note, under the light/light (L/L) cycle (12/12 hours), animals did not eat enough to be sufficiently infected by *S. aureus* compared with the L/D and D/D conditions to proceed for experiments. After feeding there was 1.32 × 10^6 less bacteria in worms under the L/L condition compared with animals under the L/D and the D/D condition (Fig. S2C). Consistent with previous studies, we found that the control *S. mediterranea* eGFP (RNAi) worms had the ability to resolve *S. aureus* infection in 6 d,\(^2\) independently of the light cycle (Fig. 2B). Worms that were subjected to the D/D cycle and knocked down for Smed-Arntl-1 and Smed-Tim eliminated *S. aureus* in 6 days, similarly to the control eGFP (RNAi). Under the L/D condition, planarians silenced for Smed-Arntl-1, eliminated *S. aureus* in 6 d (Fig. 2C). In worms silenced for Smed-Tim expression under the L/D condition, we found that the elimination of *S. aureus* was significantly delayed because it took 9 d instead of 6 d to observe the complete elimination of *S. aureus* (Fig. 2C). Indeed, at 3 d post-infection 3.5 × 10^4 ± 2.2 × 10^4 *S. aureus* CFU/animal were detected in eGFP (RNAi) control worms and 1.9 × 10^6 ± 1.3 × 10^6 *S. aureus* CFU/animal in Smed-Tim (RNAi) planarians. After 6 d of infection, bacteria were not detected in the control worms (eGFP RNAi), in contrast to the Smed-Tim (RNAi) planarians (2.3 × 10^2 ± 1.2 × 10^2 CFU/animal). At day 9, bacteria were not detected in *S. mediterranea* that had been silenced for Smed-Tim expression (Fig. 2C). To note, silencing Smed-Tim does not significantly affect the capacity of worms to ingest bacteria (1.9 × 10^7 ± 2.2 × 10^7 CFU/animal) compared with the control (1.3 × 10^2 ± 1.2 × 10^2 CFU/animal) at day 0, respectively (Fig. 2D). Thus, the delay in the elimination of *S. aureus* that was observed in Smed-Tim (RNAi) knockdown worms cannot be explained by an alteration in the ability to feed. Taken together, these data suggest that planarians living in the L/D condition require the expression of Smed-Tim to have an efficient antibacterial immune response. For the rest of the study, we focus on Smed-Tim because in contrast to Smed-Arntl-1, it plays a role in the anti-microbial response of planarians against *S. aureus*.

Third, we wondered if Smed-Tim is a component of the planarians’ circadian clock. To this purpose, we assessed the cycle of Smed-Tim expression under both conditions L/D and D/D condition (Fig. 3A and Fig. 3B). Under the L/D condition, we observed a Smed-Tim expression cycle (Fig. 3A). The increase in Smed-Tim expression is observed in the dark phase of the L/D condition between Circadian Time (CT) 12 and CT 24, and CT 36 and CT 48, with a maximum fold change of 1.20 ± 0.33 at CT 20 and 0.96 ± 0.16 at CT 44 (Fig. 3B). In contrast, under D/D condition, we observed a loss of synchronisation which lead to a phase shift called free run (Fig. 3B).\(^\text{\textsuperscript{13}}\) Interestingly, worms infected and submitted to L/D cycle had a level of Orn-Tim mRNA which showed a 2.3-fold increase at CT 20 (Fig. 3C). Then, we visualized the expression of Smed-Tim mRNA by in situ hybridization (ISH) in planarians infected or not by *S. aureus*. In the planarian tissues, we observed...
that *Smed-Tim* is weakly expressed (Fig. 3D and Fig. S3) as in several other organisms.\textsuperscript{14-17} Next, we investigated the level of production of the melatonin in planarians, a hallmark of circadian rhythm functionality.\textsuperscript{18} As described previously\textsuperscript{3} in planarians under L/D condition, we observed an increase in the production of melatonin at CT 18 (Fig. 3E), in the same concentration range than previously observed.\textsuperscript{3} In animals silenced for *Smed-Tim* we find that the melatonin level showed a significant 3-fold increase between CT 14 and 22 compared with the control animals *eGFP (RNAi)* (Fig. 3E). Taken together, these data suggest that *Smed-Tim* might play a
role in the circadian clock machinery regulation, and thus we can hypothesize that Smed-Tim is a component of the circadian clock of planarians.

Finally, we analyze whether Smed-Tim silencing affects the expression of the planarians’ antimicrobial gene responses such as p38 MAP-Kinase and Traf6, that transduces signals of pathogen perception\(^1\), and morn2, that controls the LC3-associated phagocytosis of bacteria for their destruction.\(^2\) The expression of Smed-Morn2 (Fig. 4A), Smed-Traf6 (Fig. 4B) and Smed-p38 MAPK (Fig. 4C) mRNA is significantly induced in worms from 6 hours to 36 hours following S. aureus infection, with a maximum fold change of around 4 to 12 hours post infection. The silencing of Smed-Tim diminished the mRNA expression of Smed-morn2 by 69% (Fig. 4D) and Smed-Traf6 by 75% (Fig. 4E) compared with the control animals infected by S. aureus and maintained in L/D cycle. In contrast, p38 MAP-kinase mRNA expression is not changed (Fig. 4F). To note, in planarians under D/D condition and infected by S. aureus, the silencing of Smed-Tim does not affect the expression of Smed-morn2 (Figure. S4A), Smed-Traf6 (Figure. S4B) and Smed-p38 MAP-kinase mRNA (Figure. S4C). Taken together, our data suggest that Smed-Tim regulates the capacity of planarians to kill S. aureus by modulating the antimicrobials such as Smed-Traf6 and Smed-morn2.

Discussion

In this work, we have analyzed the contribution of the circadian clock component in planarian antimicrobial activity. We have identified in planarians the homologs of human Tim and Arntl-1, however we haven’t identified homologs for Clock and Per gene. The molecular components of the planarian clock machinery may differ from those in evolved organisms such as mammals. Indeed, as previously shown in sponges, Clock, which is the main component of the circadian machinery in bilaterians, is absent from the Schmidtea mediterranea genome and transcriptome databases. Per-2 is also absent from these databases and is not found in the starlet sea anemone Nematostella vectensis, though it has a functional circadian clock that remains driven by light cues.\(^21\)
It is possible to hypothesize that *Clock* and *Per-2* genes were lost from the planarian lineage. Nevertheless, the presence of 2 others genes, *Arntl-1* and *Tim*, suggests the existence of a circadian clock machinery in planarians. This is reinforced by the capacity of planarians to produce melatonin (3 and in this study). We have investigated the implication of these 2 genes in the anti-bacterial response of planarians against *S. aureus*. In contrast to *Smed-Arntl-1*, we observed that *Smed-Tim* played a role in the anti-bacterial activities of planarians, since it silencing via RNAi delayed *S. aureus* elimination. The silencing of *Smed-Arntl-1* did not induce the death of infected worms. In other model organisms, the knock down of *Arntl-1* changed significantly the survival rate of these models during bacterial infections. Indeed, in mice, silencing *Arntl-1* prevented a decrease in the survival rate after infection with *L. monocytogenes.* Arntl-1 (−/−) fruit flies were more resistant to *Pseudomonas aeruginosa.* Therefore, the involvement of Arntl-1 during the response to bacteria could be a function of either the model organism or the bacterial species.

TIM is a key component of the circadian clock in Drosophila, but whether the mouse homolog is involved in the circadian rhythm remains controversial.
Moreover, numerous non-clock functions for timeless have been described. TIM has been reported to play a central role in the control of D. melanogaster susceptibility to bacterial infection. Indeed, TIM is required for D. melanogaster to resist Streptococcus pneumoniae and L. monocytogenes infections. A contradictory study has shown that TIM expression is deleterious when D. melanogaster is infected with P. aeruginosa. Here we unravel the idea that TIM is involved in the antibacterial potential of the flatworm under the L/D condition. The silencing of Smed-Tim delayed bacterial elimination, but the planarians remained able to eliminate bacteria. Thus, silencing Smed-Tim is not deleterious for planarians that are infected with S. aureus. We cannot exclude the possibility that Smed-Tim silencing could be deleterious or does not exert any action on planarians infected with pathogens other than S. aureus because of the pathogen-dependent actions of Tim. Interestingly, several pieces of our data suggest that Smed-Tim is might be a component of the circadian cycle of planarians. Indeed, we have observed that in L/D condition there is a cycle of Smed-Tim expression, and that Smed-Tim has a maximum expression in the dark phase compared with what is observed in anopheles species and drosophila.

The production of melatonin has been defined as a hallmark of circadian rhythm functionality, and a perturbation of the circadian clock machinery lead to a dysregulation of the melatonin production. We do not
observe an inhibition of melatonin production in animals silenced for Smed-TIM as expected. However, we find a deregulation of the melatonin production in animals silenced for Smed-TIM, characterized by an increase in the level of melatonin production in planarians, allowing us to hypothesize a potential link between Smed-Tim and circadian clock in planarians. Finally, we analyze whether Smed-Tim silencing regulates the expression of the antimicrobial genes of planarians, such as p38 MAP-kinase and Traf6, that transduces signals of pathogen perception,19,20 and morn2, that controls the LC3-associated phagocytosis of bacteria for their destruction.2 Importantly, we characterized the critical involvement of Smed-Tim in the induction of Smed-Traf6 and Smed-morn2 mRNA expression in response to S. aureus in the L/D condition. Interestingly, in the D/D condition; condition in which Smed-Tim cycle is in free run, the planarians’ antimicrobial genes tested here are not regulated by Smed-Tim. This underscores the importance of a 12/12 Light and dark cycle in the anti-microbial capacity of planarians and that Smed-TIM is in upstream of Smed-TRAF6 and Smed-MORN2.

In conclusion, here we identified the planarian homolog to Hs-Tim and revealed that Smed-TIM is a gene of the circadian cycle of planarians, and that Smed-Tim regulates the expression of downstream antimicrobial genes such as Smed-morn2 and Smed-Traf6 expression during S. aureus infection.

Methods

Materials and methods

Planarians

Planarians belonging to the species Schmidtea mediterranea (CIW4) were used. Planarians were maintained in static culture as described previously26 in autoclaved water at 20°C and fed once per week with calf liver. Animals were starved for at least 1 week before the experiments. Water was changed every 2 days, and planarians were maintained without antibiotics. Before experiments, worms were subjected to the L/D cycles (12/12 h) for 4 weeks.

Bacteria

Staphylococcus aureus (ATCC25923) was grown on blood agar plates (BioMerieux SA) at 37°C.

Worm feeding with bacteria

S. mediterranea were fed with S. aureus using a protocol adapted from a dsRNA feeding method27 as described previously.2 Briefly, S. aureus (1 × 10^9) were suspended in homogenized liver, mixed with ultra-low-gelling-temperature agar and red food coloring, and allowed to solidify on ice. Room-temperature, solidified food was fed to planarians. After 2 hr (defined as day 0) of feeding, the planarians were extensively washed.

CFU counting

As described previously,2 S. mediterranea were collected and homogenized in PBS. The lysate was passed 5 times through a sterile syringe with a 29G needle to disrupt planarian tissue clumps, and CFUs were counted after plating 10 µl of serial dilutions onto blood agar plates (BioMerieux SA).

Gene prediction

To identify a planarian homolog of Hs-ARNTL-1 (NP_001025443.1, NM_001030272.2), Hs-Per-2 (NP_0073728, NM_022817.2), Hs-Tim (NP_003911.2, NM_003920.3), and Hs-CLOCK (NP_001254772.1, NM_001267843.1) we examined, via TBLASTN (e-value 1e^-5), the S. mediterranea transcriptome database PlanMine (http://planmine.mpi-cbg.de/planmine/begin.do)10 and the S. mediterranea genome database SmedGD (http://smedgdkc03.stowers.org/).11 We identified sequences producing a significant alignment with the genes of interest. The top BLAST hit was used to predict Smed-Tim and Smed-Arntl-1 via FGENESH+ (http://www.softberry.com/). Homology at the protein level between predicted Smed-Tim and Hs-Tim, or Smed-Arntl-1 and Hs-Arntl-1 was analyzed using BLAST (see supplemental data online for predicted sequence). The conservation scoring was performed by PRALINE (http://www.ibi.vu.nl/)12 using default parameters. The results are color-coded for amino acid conservation and the scoring scheme works from 0, for the least conserved alignment position, to 10, for the most conserved alignment position. Nuclear localization sequence in Smed-TIM was identified using cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) with the following setup: cut of score 6.

Cloning

To generate the Smed-Tim and Smed-Arntl-1 RNAi molecules, cDNA from S. mediterranea was amplified via PCR using primers Tim (left primer TGGA-GAGTGGCTGGAAGGAT, right primer CGAA-GAATGGCCGACTCTG), Arntl-1 (left primer CAGAAGGTACCACGACCGAT, right primer TGAT-GAGTGGCTGGAAGGAT, right primer CGAA-GAATGGCCGACTCTG), Arntl-1 (left primer CAGAAGGTACCACGACCGAT, right primer TGAT-GAGTGGCTGGAAGGAT, right primer TGAT-GAGTGGCTGGAAGGAT), and Primer3 (http://primer3.sourceforge.net/) and containing attB recombination sequences (CATTACCATCCCG). PCR products were cloned17 and sequences verified on a 3130XL Applied machine, and Blast against the predicted gene sequence. The dsRNA in silico accuracy
prediction was defined as follows. Targeted transcript sequences were extracted between the 3’ end of the 5’ primer and the 5’ end of the 3’ primer used for cloning. The extracted sequences were then cut into 21 mers using a sliding window of 1 nucleotide. All possible RNAi sequences for Smed-Tim and Smed-Arntl-1 were then generated, and each putative RNAi sequence was aligned to the planarian transcriptome using BLAST28 with a word size of 21; only perfect matches were considered. For each transcript for which an RNAi was designed, theoretical target accuracy was calculated based on the number of RNAi sequences matching the target divided by the total number of generated RNAi sequences. The number of theoretical off-target events was equal to 0, thus giving a target accuracy of 100%, which strongly suggests a gene-specific effect but does not exclude the possibility of an off-target effect (Table. S1 and S2).2

**Delivery of dsRNAs**

dsRNAs were delivered to S. mediterranea as described previously.2,27,29 Briefly, animals were submitted to 3 rounds of RNAi feeding (1 every 3 days), then 3 d after the last RNAi feeding, worms were challenged by S. aureus.2 The quality of Smed-Arntl-1 and Smed-Tim knock down was controlled via Real-time RTqPCR as described elsewhere.2,30 Primers used for RTqPCR were for Smed-Arntl-1 (left primer ACGTGGAATGTGTAATGCTG, right primer AAAACCATCAGCAGCCTCCA) and for Smed-Tim (left primer GGATCTTGGGACGGCCGATT, right primer AATCCCGGTTTTCCCGGCC). The expression of the Smed-p38 MAPK, Smed-Traf6, and Smed-morn2 was evaluated via RTqPCR using the following primers: Smed-Morn2 (CGTCAAGGGAAAGGTATTAGCG, GTCGCCTTCAATTGTGACCA), Smed-p38 MAPK (GCGAGGCA- GACGATGGAAGA, GCGTGTAAACAATTCGGCCA), and Smed-Traf6 (CGTCAAGGGAAAGGTATTAGCG, GTCGCCTTCAATTGTGACCA), and Smed-Traf6 (ACCCCAACTCAATAGGCA, AACTCCATTGTGCCCAGGT).

The results were normalized by the expression of the control housekeeping gene Smed-ef2.31

**In situ hybridization and probe synthesis**

Whole-mount *in situ* hybridization was performed as described previously.32,33 All animals were 1 to 2 mm in length and size-matched between the experimental and control groups for S. mediterranea infection. The animals were imaged using a Leica M165FC stereomicroscope (Leica, Heidelberg). The images were processed using Adobe Photoshop CS5 software, and figures were assembled using Adobe Illustrator Artwork 15.0.

**Melatonin assay**

The melatonin level was determined using Melatonin ELISA KIT (Emelca Bioscience). Briefly, worms (30 per time point, size 10 mm to 7 mm) were lysed in phosphate buffer salt solution complemented with proteases inhibitors (Roche), then samples were done according to the manufacturer’s recommendation.

**Statistical analysis**

The results are expressed as the mean ± SD and were analyzed using the nonparametric Mann-Whitney U test. Differences were considered significant at p < 0.05.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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