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A real-time analysis of QacR-regulated multidrug resistance in *Staphylococcus aureus*☆

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Abstract

Here we describe the construction and characterization of a biosensing reporter where *luxCDABE* genes from *Photorhabdus luminescens*, engineered for expression in Gram-positive organisms, are under the transcriptional control of QacR repressor from *Staphylococcus aureus*. In non-pathogenic *S. aureus* model system we analyzed the activity of the regulatory region acting as multidrug-resistance mediator in wild type strains. The use of full-length bacterial luciferase and the measurement of real-time light emission from intact, living cells make the present system suitable to follow the short term activity of different inducers. Among the tested molecules, tetracyclines showed a peculiar behavior by giving very high induction in a fashion seemingly more related to a general stress condition of the culture than to the direct binding and displacement of QacR from its operator. Temperature shocks confirmed that active transcription from *qacA* promoter is started in response to unspecific conditions where cell growth is strongly inhibited.

Keywords: *S. aureus*; Multidrug resistance; Membrane efflux pumps; QacA; QacR; Quaternary ammonium compounds; Lipophilic cations; Tetracyclines; Biosensor; Luciferase

Proton motive force-dependent efflux of toxic compounds has been demonstrated to mediate multidrug resistance in different bacterial species [1,2]. Such kind of resistance is of crucial importance when pathogenic strains [3,4] are involved; consequently the export systems' components should be carefully considered as targets for antimicrobial therapy [5]. For *Staphylococcus aureus*, which plays a major role in hospital-borne infections [3], many plasmid-encoded multidrug resistance transporters [6,7] have been described.

QacA makes *S. aureus* resistant to various toxic organic cations [8,9] and its expression is tightly regulated [10–12] by the divergently encoded QacR repressor protein [13–15]. QacR belongs to the family of TetR/CamR transcriptional regulators [12,13] and binds to a 28-bp inverted repeat (IR1) lying between *qacA* and *qacR* coding sequences and overlapping the transcriptional start of *qacA* gene [13]. The repressor protein differs slightly from other members of the family in binding the operator as a pair of dimers and in the length of the operator itself [15]. When bound to the major groove of the DNA QacR prevents the transition of RNA polymerase-promoter complex into a productively transcribing state [14]. The binding of one of many structurally dissimilar lipophilic compounds [8,9], substrates for QacA membrane pump, displaces the repressor from the operator site thus initiating transcription [8].

The development of a tool to follow the short term induction of QacR-regulated genes was the aim of the present study. To achieve this task the regulatory region

☆ *Abbreviations:* IR, inverted repeat; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; bp, base pair(s); MCS; multiple cloning site; QAC, quaternary ammonium compound; OD_{595}, optical density at 595nm; OD_i, OD_{595} in presence of a putative or known inducer; OD_c, OD_{595} in negative control wells; CPS, counts per second; CPS_i, CPS in presence of a putative or known inducer; CPS_c, CPS in negative control wells; IC, induction coefficient; IC_2, induction coefficient normalized with OD_{595}.

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and the divergent *qacR* coding sequence from the staphylococcal multiresistance plasmid pSK1 [6] are coupled to the modified bacterial luciferase operon from *Photorhabdus luminescens* [16] and the resulting plasmid, pQacLux, was used as a biosensing element in non-pathogenic *S. aureus* RN4220 strain [17,18]. The use of real-time reporter system and the expression in the species naturally hosting QacA multidrug transporter are the issues on which attention is especially focused. Previously characterized systems, in fact, are all based on time-consuming CAT assays and shown to work only in *Escherichia coli* [14,15].

Real-time measurements based on bioluminescence, after the addition of known inducers, avoid the need for cell lysis and quickly provide reliable data that fully match previous knowledge. In properly arranged systems the background gene expression and the reporter activity are negligible and therefore it is possible to ascertain the sometimes weaker influence of previously untested molecules, giving an additional insight into how *S. aureus* cells answer to different chemical and physical stimuli in terms of efflux pumps expression.

Materials and methods

Bacterial strains and plasmids. All bacterial strains, plasmids, and primers used in the present study are described in Table 1. Cloning and selection of the recombinant plasmids were performed in *E. coli*, strain Xl1-blue. Non-pathogenic *S. aureus* strain RN4220 was used for the expression of pQacLux and for studies concerning QacR-regulated expression of light-emitting protein reagents. All strains were cultured in either liquid or semi-solid LB media, complemented, when appropriate, with ampicillin 100 μg/ml, chloramphenicol 10 μg/ml or gentamicin 20 μg/ml.

DNA manipulations, including plasmid isolation and molecular cloning, were performed by standard methods, as described previously [19]. Staphylococcal multiresistance plasmid pSK1, isolated from pathogenic *S. aureus* strain SK982, was the original source for *qacR* regulatory region. PCR using the primers P1 and P2 was used to generate a 764 bp fragment, including divergently transcribed *qacR* coding sequence and *qacR–qacA* intergenic region (744 bp) within *Nco*I (5′) and *Apa*I (3′) sites. *luxABCDE* operon (5619 bp) was excised from pSB2025, provided by Dr. Philip J. Hill, by means of *Nco*I (5′) and *Pst*I (3′) restriction enzymes. The resulting 5703 bp fragment contained the reporter cassette and some additional nucleotides from cloning vector pSL1190 MCS [16], which encoded also for an *Apa*I site lying 39 bp upstream of *luxA* gene. The excision product was ligated into *Nco*I–*Pst*I restricted pNZ8048, provided by Dr. Oscar P. Kuipers, producing pA000, where *lux* genes are downstream of *PnisA*, nisin inducible promoter from *Lactococcus lactis* [20].

Cloning of the entire *qacR–qacA* regulatory region into *Nco*I–*Apa*I restricted pA000, immediately upstream of the reporter cassette, finally produced pQacLux (see Fig. 1).

Induction assays. All assays were performed in white-walled, transparent-bottomed multi-96 microtitration plates (Nalge Nunc International, USA), allowing the measurement of absorbance at 595 nm and luminescence (integral measurement, 1.0 s) at the same time. Readings were performed by means of Wallac Victor 1420 microtitration plate multilabel counter (Perkin-Elmer, Turku, Finland). Putative or known inducers at different concentrations (ranging from 0.4 ng/ml to 0.4 mg/ml) were added to *S. aureus* RN4220/pQacLux cells in their early logarithmic phase (typically with an OD_{595} of about 0.06) directly in the plate wells. Pure, commercial water (Pharmacia, Uppsala, Sweden) was used to provide required negative controls. After the addition, light emission was measured every 60 min (30 min when shorter time-frame information was required), during which the plate was incubated at 37 °C under vigorous shaking (900 RPM, Thermo Labsystems Plate Shaker, Helsinki, Finland). Absorbance was routinely checked only at the beginning and at the end of the assays, which lasted a maximum time of 360 min. The chloramphenicol selective

Table 1
Strains, plasmids, and oligonucleotide primers

Strain/plasmid/primer	Genotype/features/sequence	Ref./Source
Strain[a]		
RN4220	*S. aureus*; restriction-deficient derivative of 8325-4 (r_k^- m_k^+)	[17,18]
SK982	*S. aureus*, DSMZ No. 9385; multidrug resistant laboratory strain, $pSK1^+$	[24] DSMZ
Xl1-blue	*E. coli*; *endA1*, *gyrA96*, *hsdR17* (r_k^- m_k^+), *lac*, *recA1*, *relA1*, *supE44*, *thi-1* [F′*proAB*, *lacI*^{q}*ZΔM15*, *Tn10* (Tet^r)]	[25] Stratagene
Plasmid[b]		
pSK1	*S. aureus* multiresistance plasmid conferring gentamycin, trimethoprim and multidrug resistance	[6]
pNZ8048	3349 bp; shuttle vector replicating in *E. coli* and *S. aureus*; MCS downstream of *PnisA*; Cm^r	[26]
pSB2025	*luxABCDE* operon cloned into superlinker plasmid pSL1190, Ap^r	[16]
pA000	9043 bp, *luxABCDE* excised from pSB2025 and cloned into pNZ8048 MCS, Cm^r	This study
pQacLux	9755 bp, *qacR–qacA* regulatory region from pSK1 ligated into pA000 upstream of *luxABCDE*, Cm^r	This study
Primer[c]		
P1	5′-TATT*CCATGG*TTATTTACTAAGTCCATTTA-3′	
P2	5′-TATT*GGGCCC*TTAAGTTCCCTCCAATCCTT-3′	

[a] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; Stratagene, Stratagene Co., La Jolla CA 92037, USA; Tet^r, tetracycline resistance.

[b] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; MCS, multiple cloning site; *PnisA*, nisin inducible promoter from *L. lactis*. For more detailed information about plasmid construction please refer to "Materials and methods."

[c] Additional nucleotides for the incorporation of restriction sites and for subsequent direct cleavage are underlined; inserted restriction sites are in italic.

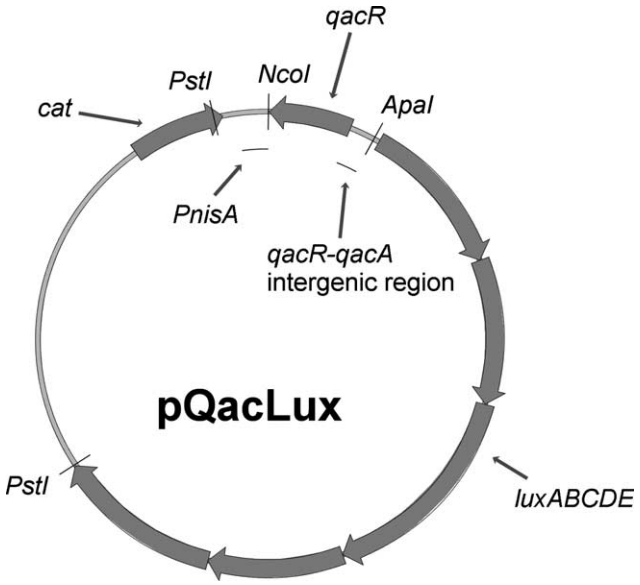

Fig. 1. pQacLux map. Relevant features of the 9755 bp pQacLux plasmid are depicted. Restriction sites used during cloning steps are reported. *luxABCDE* constituting genes and *qacR* coding sequences are schematized as arrowed box, to underline their transcriptional divergence; *cat* transcriptional direction is reported, as well. *PnisA* promoter and *qacR–qacA* intergenic region can be identified by means of small bars flanking their whole length. For more detailed information about plasmid construction please refer to "Materials and methods."

pressure was kept for the whole procedure. Each reading entailed three parallel replicates.

Thermal shocks. One ml aliquots of pQacLux bearing RN4220 cells in their early logarithmic phase ($OD_{595} = 0.06$) were transferred into microcentrifuge tubes and thermally shocked. Cold shock (+4 °C, 10 min) and heat shock (+54 °C, 10 min) [21] were administered and subsequently cells were moved back in measurement-suitable plates. Control tubes were kept at room temperature for the same time. Each assay entailed three parallel replicates. Culture recovery and luminescence measurements were performed as described under "Induction assays" for chemicals addition.

Data handling. The induction, possibly given by selected compounds, was monitored along the whole assays in terms of CPS values, optical density, and ICs. CPS, giving a direct estimate on the level of light emission, were obtained in output from the counter and averages among the three replicas were calculated. ICs resulted by dividing the level of luminescence measured in treated wells (CPS_i) by the level recorded in control wells (CPS_c). Kinetic curves were plotted with time in abscissa and either CPS or ICs in ordinate. In addition, specific time points along the assay were chosen to plot ICs against the inducer concentration (curves not reported). Concentrations able to give a maximal induction were identified for all molecules taken into consideration and values were compared, where possible, with previous data.

When chemicals showed a strong inhibitory activity upon cell growth a second mathematical operator, named IC_2, was introduced in the attempt to normalize IC with the effective cell number. The aim was to get an idea on the extent to which single cells are induced. IC_2 was calculated by multiplying IC with the ratio between OD_c and OD_i. If no growth inhibition occurs the mentioned ratio is equal to 1 and as a consequence IC and IC_2 completely match each other. IC_2, when available, was used as IC in plotting kinetic and induction-concentration curves. It should be noted that neither IC nor IC_2 requires units of measurements, being calculated as ratio between physical amounts.

Results

Previously described inducers are able to start light emission from pQacLux

Model compounds were selected from each class of molecules whose activity toward *qacR–qacA* regulatory region was already demonstrated [14] and they were tested as described under "Induction assays." In addi-

Table 2
Induction of light emission from pQacLux upon the addition of various known and previously untested inducers

Compound	Class[a]	Concentration responsible of the maximal induction	IC[b]
Known inducers[c]			
Chlorhexidine digluconate	Biguanidine	0.8 μg/ml	17
Pentamidine isethionate	Diamidine	20 μg/ml	106
Crystal violet	Dye	60 ng/ml	22
Ethidium bromide	Dye	8 μg/ml	17
Malachite green carbinol base	Dye	0.4 ng/ml	6
Benzalkonium chloride	QAC	1 μg/ml	520
Cetylpyridinium chloride	QAC	0.4 μg/ml	12
Tetracyclines[d]			
Demeclocycline hydrochloride	Tetracycline	20 ng/ml	314
Methacycline	Tetracycline	40 ng/ml	302
Oxytetracycline hydrochloride	Tetracycline	100 ng/ml	257
Tetracycline hydrochloride	Tetracycline	60 ng/ml	132

[a] QAC, quaternary ammonium compound.
[b] IC, induction coefficient. Induction coefficients were calculated by dividing the level of luminescence obtained with the addition of inducers by the level obtained in absence of inducing compounds. Reported ICs are the highest obtained for each compound; values are rounded to the first integer figure. Luminescence measurements and induction assays were performed as described under "Materials and methods."
[c] ICs refer to 240 min after the inducer administration.
[d] ICs refer to the end of the assay (360 min).

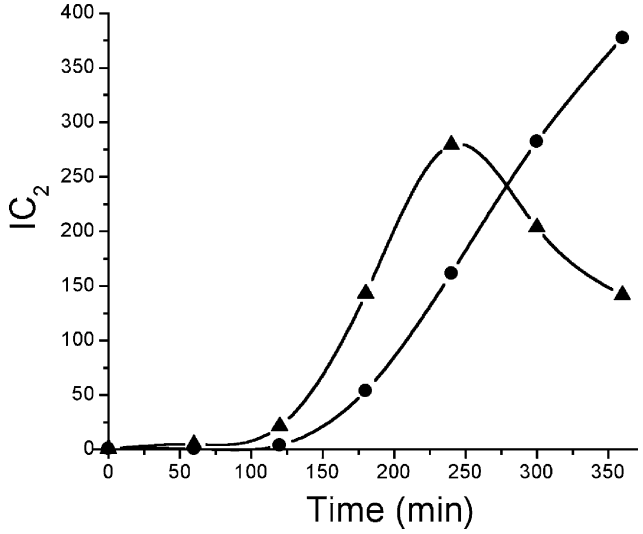

Fig. 2. Dual pattern of light emission from pQacLux. Benzalkonium chloride (▲) and tetracycline hydrochloride (●) were administered to exponentially growing RN4220/pQacLux cells at time 0. Maximally inducing concentrations (1 μg/ml and 60 ng/ml, respectively) were used. Assays and data handling were performed as described under "Materials and methods."

tion various other compounds were tested to assess the specificity of the system. Table 2 summarizes the results for those treatments where induction occurred. Ampicillin, ammonium and guanidine salts, erythromycin, chloramphenicol, and gentamicin were not able, in the wide concentration range used for the assays, to drive transcription from *luxABCDE* operon (data not shown).

When the appropriate concentration of inducer is administered to the growing cells light emission rapidly starts and the cell density depending factor IC_2 keeps on raising for about 240 min. Thereafter the signal from treated wells moves to a decaying trend while negative control wells give almost constant luminescence for the whole assay time. Fig. 2 depicts the described induction behavior for benzalkonium chloride. All compounds mentioned in the first section of Table 2 act on pQacLux with the same fashion, though to different extents.

Tetracycline induction

In the attempt to ascertain the specificity of the system also tetracyclines were tested. Unexpected strong induction occurred but the time course of light emission differed considerably from other inducers. As depicted in Fig. 2 for tetracycline hydrochloride, luminescence starts with a slight delay in comparison to benzalkonium chloride. Furthermore no regression of the signal occurs and the curve keeps on raising until the end of the assay. The latter section of Table 2 reports data concerning different tetracyclines; they all behave as described for tetracycline hydrochloride in terms of kinetic induction (data not shown).

Tetracyclines, at concentrations used in this study, quickly inhibit cell growth when added to RN4220/pQacLux cells in plate wells, as monitored with OD_{595} measurements. This kind of inhibition is very fast, appearing already after 30 min, and it witnesses the entrance of the antibiotic molecules into the cytoplasmic environment, where the targets for their antimicrobial activity, namely ribosomes, are. On the contrary the observed induction is delayed, starting faintly after 120 min, even though it subsequently shows a steep trend until the end of the assay (see Fig. 5).

Luminescence as response for thermal stress

Since tetracycline induction gave an idea of some kind of unspecificity regarding QacR-regulated expression, we also tested few other stress conditions. In the present section the difference between IC and IC_2 plays its most important part and should be kept carefully in mind (please refer back to "Data handling" for details). Fig. 3 depicts the kinetic luminescence answers obtained after the administration of cold and heat shocks as described under "Thermal shocks." Cold shock does not start light emission from the system; cells recover quickly and IC_2 is constantly around 1. IC in this case is slightly lower (data not shown) because growth is weakly affected by the thermal treatment.

Heat shock, on the other side, results in a hard stress condition for the culture. Cells do not replicate for the first 180 min (OD_{595} remains still around 0.07) and the recovery is quite slow even thereafter. Nevertheless during the recovery phase luminescence is moderately

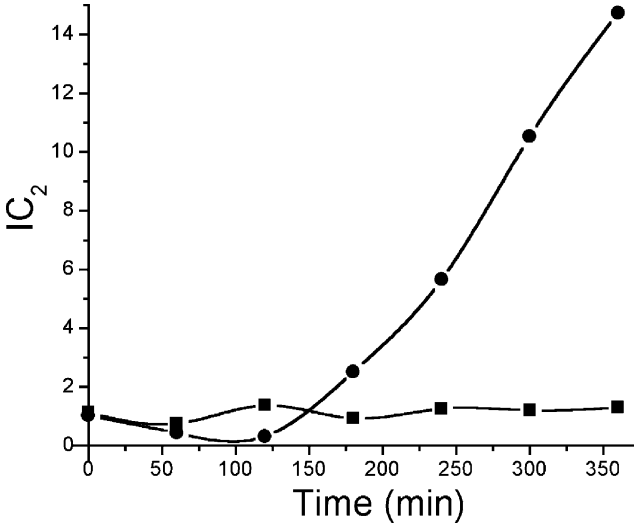

Fig. 3. Luminescence induction upon thermal shocks. RN4220/pQacLux cultures in their early-mid logarithmic growth phase were kept at 54 °C (●) and at 4 °C (■) for 10 min immediately preceding the initiation of the assay. Negative control cultures were kept for the same time at room temperature. Assays and data handling are detailed under "Materials and methods."

induced, in coincidence with the resumption in OD_{595} increase. IC_2 steeply raises in a fashion similar to the one recorded with tetracyclines, though in much lower extent. Here IC and IC_2 differ considerably (data not shown) because of the large gap between shocked and control cultures in terms of cell number.

In parental control plasmid pA000 specific induction is completely abolished while tetracycline-mediated and shock-related light emission is only reduced

The plasmid pA000, containing no *qacR* regulatory region within the MCS, was submitted to the same assays which were originally arranged for pQacLux, as described under "Materials and methods." As expected, tested QACs, diamidines, biguanidines, and dyes showed to be completely unable to start *luxABCDE* expression from the plasmid, thus confirming the crucial role played by *qacR–qacA* regulatory region in specific induction. Surprisingly the answer of the system to heat shock (data not shown) and tetracyclines was not totally abolished but strongly reduced. Fig. 4 compares the kinetics of light emission from pQacLux and pA000 in presence of tetracycline hydrochloride, 60 ng/ml. It is evident how the curves match each other in terms of the raising trend and differ only with regard to the extent of light production. The signal from pQacLux is, in fact, about 5-fold higher when the same concentration of tetracycline is administered to both plasmids. Nevertheless ICs around 55 were recorded also for pA000, where *PnisA* promoter, showing neither activity nor considerable leakage in *S. aureus* under normal culture conditions [20], is controlling *lux* cassette expression.

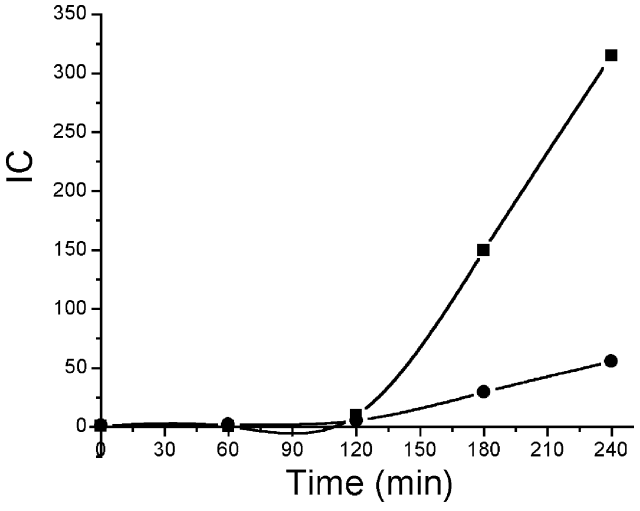

Fig. 4. Comparison between the parental plasmid pA000 and the biosensing plasmid pQacLux with regard to tetracycline induction. Tetracycline hydrochloride, 60 ng/ml, was added to exponentially growing RN4220/pQacLux (■) and RN4220/pA000 (●) cells at time 0. Please refer to "Materials and methods" for more detailed description of assay setup and data handling.

This strongly suggests that *qacR–qacA* regulatory region in this case acts only in amplifying a response, involving different closed promoters, whose origin lies elsewhere.

Discussion

The present work provides the first experimental evidences to demonstrate that QacR-repressed transcription can be differently induced, depending on the inputs received by the cells. A first, previously demonstrated [14], short-term induction occurs when specific compounds (Table 1, 1st section) enter the cell, bind QacR, and displace it from IR1 operator [8]. All these compounds, though structurally dissimilar and belonging to different chemical classes, are substrates for QacA membrane efflux pump [7–9] and are actively exported when their concentration rises within the cell. High-level production of QacA protein in response to these chemicals directly leads to a condition of increased multiple resistance [7,9], with regard to the actual inducer and to all other molecules exported by the system.

Inducers act in driving multidrug resistance gene transcription at fractional inhibitory concentrations [9]. A slight effect on cell growth is thus observed also during assay runtime (data not shown). The faint inhibition of bacterial replication starts immediately after the access of the chemical to the cytoplasmic environment and it coincides with the light production from pQacLux bearing *S. aureus* RN4220 cells. This proves the molecule under investigation to be specifically responsible for the induction.

Tetracyclines produce a different response, seemingly closer to the one recorded by means of heat shock. Transcription from *luxABCDE* operon upon tetracycline addition is, in fact, delayed and it shows a continuously rising trend (see Fig. 2). This is also the case observed upon heat treatment (see Fig. 3). Furthermore Fig. 5 depicts clearly how the tetracycline entrance in the cytoplasm does not result in immediate light emission as it occurs for other inducers. Cell growth is very soon inhibited while luminescence is started only 2 h after the antibiotic molecules are added to the culture.

The reaction to tetracyclines is similar to heat shock recovery also for what concerns the parental plasmid pA000, where the lack of the entire *qacR–qacA* regulatory region does not abolish the induction recorded consequently to either kinds of treatment. In pA000 *lux* operon is placed under the transcriptional control of *PnisA* promoter from *L. lactis* [20]. *PnisA* is normally activated only in *L. lactis* by an extracellular signaling pathway related to quorum sensing when sub inhibitory concentrations of nisin exist in the medium [20]. Different proteins, whose coding genes are missing from both pNZ8048 and pA000, are required for the

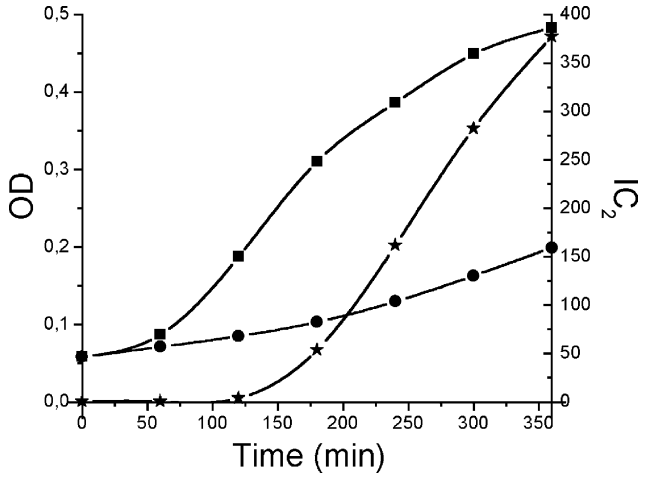

Fig. 5. Tetracycline treatment results in immediate growth inhibition but in delayed unspecific induction of light emission. Tetracycline hydrochloride, 60 ng/ml, was added at time 0 to growing RN4220/pQacLux cells. Pure, commercial water was used in place of the antibiotic for negative control experiments. OD_i (●) and OD_c (■) refer to left *Y* axis. IC_2 (★) refers to right *Y* axis. Assays and data handling were performed as described under "Materials and methods."

nisin-mediated induction of *PnisA* in *S. aureus*, as demonstrated by Eichenbaum et al. [20]. No activation of this promoter is, therefore, possible in *S. aureus*, if missing functions are not additionally provided. Downstream transcription from *qacR–qacA* regulatory region is also prevented when inducers are not administered, because of the steady repression due to QacR [13].

Tetracyclines and heat shock demonstrated the ability to unspecifically or indirectly circumvent the transcriptional repression given by QacR and to activate *PnisA*, even in the absence of the required signal transduction machinery (see Fig. 4). Presumably they both act via the establishment of a general stress condition in the culture, either of chemical or physical kind, which in turn enhances leakage from many different closed promoters by means of some unidentified pathway. Candidate pathways for such a broad change in gene expression pattern could be related, for instance, to a shift of RNA-polymerase specificity determined by the recruitment of a different accessory factor, whose production is activated by the stress condition itself. Literature reports various examples of stress-induced factors in *S. aureus*, the main among them being the transcriptional regulator σ^B [22], directly responsible for starvation [23] and environmental stress survival [21]. The difference in the induction level obtained with pA000 and pQacLux (Fig. 4) upon chemical and physical stresses can be attributed to the presence of the additional *qacA* promoter in pQacLux, which provides a further docking site and an active transcriptional starting point for the RNA-polymerase complex, once the repression is avoided.

Interestingly tetracycline-mediated chemical stress completely abolishes the specific short-term response to other inducers, such as QACs, diamidines, biguanidines, and dyes, when multiple treatments are arranged. As represented in Fig. 6, benzalkonium chloride and tetracycline hydrochloride were tested at sub-maximally inducing concentrations (0.8 μg/ml and 40 ng/ml, respectively) either one by one or in association. The dual treatment, once the stress condition was entirely established, resulted in luminescence trend almost identical to the one obtained by means of tetracycline single induction.

Unspecific transcriptional activation from *qacR–qacA* regulatory region and from other regulated operators in stressed *S. aureus* cells could represent a very general route for recovery from toxic insults. To our knowledge this is the first report on unspecific induction from QacR-regulated multidrug transporter regulon and the phenomenon waits more detailed studies to show what the consequences are in global regulation of *S. aureus* gene expression.

When bacteria encounter threatening conditions their reaction is presumable to be addressed to change more and more variables, via gene expression, as the complete death of the population approaches. This fight for survival against dangerous environmental stimuli has already been demonstrated to involve specific gene regulation [23], but there is a rationale in believing that also a broader and less targeted change in the transcriptional pattern could be part of the response. The concerned mechanisms are still unidentified and they

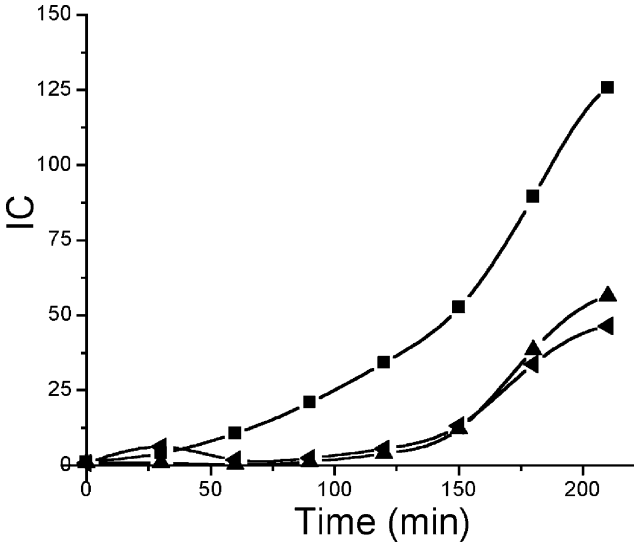

Fig. 6. The establishment of a stress condition abolishes specific induction. Benzalkonium chloride, 0.8 μg/ml (■) and tetracycline hydrochloride, 40 ng/ml (▲) were added at time 0 to RN4220/pQacLux cells and assayed as detailed under "Materials and methods." A double induction assay was performed to test the two associated compounds' activity (◀).

represent interesting candidates for further studies. The complete characterization of a general rescuing pathway for chemical and physical stresses would improve our understanding in how resistant strains evolve and are selected. Furthermore the identification of all the machinery components would provide novel targets upon which to develop drugs able to avoid, or at least inhibit, the generation of multidrug resistance.

Acknowledgments

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