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Antibiotic pressure can induce the viable but non-culturable state in *Staphylococcus aureus* growing in biofilms

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Objectives: Staphylococcal biofilms are among the main causes of chronic implant-associated infections. We have recently suggested that their transformation into viable but non-culturable (VBNC) forms (i.e. forms capable of resuscitation) could be responsible for the recurrent symptoms. This work aims to establish whether *Staphylococcus aureus* biofilms can give rise to VBNC forms capable of being resuscitated in suitable environmental conditions, the role of different stressors in inducing the VBNC state and the conditions favouring resuscitation.

Methods: *S. aureus* 10850 biofilms were exposed to different concentrations of antibiotic (vancomycin or quinupristin/dalfopristin) and/or to nutrient depletion until loss of culturability. The presence of viable cells and their number were examined by epifluorescence microscopy and flow cytometry. Gene expression was measured by real-time PCR. Resuscitation ability was tested by growth in rich medium containing antioxidant factors.

Results: Viable subpopulations were detected in all non-culturable biofilms. However, viable cell numbers and gene expression remained constant for 150 days from loss of culturability in cells from antibiotic-exposed biofilms, but not in those that had only been starved. Resuscitation was obtained in rich medium supplemented with 0.3% sodium pyruvate or with 50% filtrate of a late-log culture.

Conclusions: Our findings demonstrate that *S. aureus* can enter the VBNC state in infectious biofilms. The presence of vancomycin or quinupristin/dalfopristin can inadvertently induce a true VBNC state or its persistence in *S. aureus* cells embedded in biofilms, supporting previous findings on the role of staphylococcal biofilms in recurrent infections.

Keywords: recurrent infections, vancomycin, quinupristin/dalfopristin

Introduction

Staphylococcal biofilms are responsible for a wide range of implant-related infections as well as for most chronic ones.[1–3] In particular, 'chronic polymer-associated infections' pose a risk to human health because they are often difficult to eradicate.[1] Resistance to eradication has been attributed to factors that include the presence of biofilm-associated extracellular polymeric substances as well as biofilm-specific microbial physiologies characterized by tolerance of antimicrobials.[1,4]

In biofilms, staphylococci have been documented as persister cells and small colony variants;[5] in contrast, no data are available on their ability to enter the viable but non-culturable (VBNC) state. Such a state, characterized by low levels of metabolic activity and failure to grow on routine bacteriological media, has already been reported for several human pathogens.[6] A key feature is that appropriate stimulation can restore full metabolic activity and culturability, a process called resuscitation.[5,7]

Several factors can induce the VBNC state, including starvation, bacterial incubation outside the normal temperature range, higher or lower osmotic and oxygen concentrations, common food preservatives and heavy metals.[6–8] Achievement of the VBNC state by *Staphylococcus aureus* has been described only once, in the presence of typical environmental stress conditions (natural seawater at 4°C).[9] We have recently suggested that *S. aureus* might undergo a similar transformation in central venous catheter (CVC)-associated biofilms, where it might thus become responsible for recurrent infections upon resuscitation.[10]

This work was devised to: (i) explore the stimuli that can induce *S. aureus* biofilms to give rise to VBNC forms in conditions

similar to those found in medical device-associated biofilms; and (ii) test the ability of *S. aureus* VBNC cells to revive in suitable microenvironmental conditions and recover full metabolic activity and culturability.

Materials and methods

Bacterial strains, media, antibiotics and enzymes

The biofilm producer *S. aureus* 10850 was used in VBNC induction assays.[11] It was routinely grown in tryptic soy broth or agar (TSB and TSA, respectively; both from Oxoid, Basingstoke, UK) supplemented with 1% (v/v) glucose (TSBG or TSAG, respectively) to obtain optimal biofilm production. M9 minimal medium without glucose was used as non-nutrient (NN) agar in VBNC induction assays. The following antibiotics and enzymes were used: vancomycin (Hospira, Lake Forest, IL, USA); quinupristin/dalfopristin (Sanofi-Aventis, Milan, Italy); lysozyme, lysostaphin, RNase and proteinase K (all from Sigma–Aldrich, St Louis, MO, USA); and SmaI nuclease (Fermentas, Milan, Italy).

In vitro biofilm production, stress exposure and culturability assays

For biofilm development, 100 μL of a late-log culture of *S. aureus* 10850 grown in TSBG was spotted on 0.22 μm sterile nitrocellulose filters (Millipore Corporation, Billerica, MA, USA) and incubated in TSAG for 48 h at 37°C. The filters were then moved to NN agar plates unsupplemented or supplemented with different concentrations (MIC or 4×, 8× or 16× MIC) of antibiotic (vancomycin or quinupristin/dalfopristin) and kept at 37°C until loss of culturability. Filter cultures were transferred weekly to fresh agar plates containing identical substrates (no antibiotic or the same antibiotic concentrations) as the spent media. Culturability was assessed every 2 days by placing a loop of the filter cultures in rich medium (TSB and TSA) and incubating at 37°C for 48–72 h. All assays were performed in triplicate.

Biofilms testing negative on culturability assays were placed in 5 mL of saline, detached by three cycles of sonication (60 s) (UltraSonic Cleaner, Soltec, Milan, Italy) and vortexing (15 s), washed and then resuspended in 5 mL of saline. Finally, 200 μL was inoculated in 2 mL of TSB and incubated for 48–72 h at 37°C to confirm the non-culturable state; the remaining suspension was stored at −80°C.

Epifluorescence microscopy and flow cytometry

Epifluorescence microscopy[10] and flow cytometry[12] counts were performed as described previously.

Real-time RT–PCR assays

Total RNA was extracted from 3 mL of each detached bacterial film using the RNeasy Mini Kit (Qiagen, Hilden, Germany); the lysis buffer was supplemented with lysozyme (10 mg/mL) and lysostaphin (250 μg/mL). Samples were incubated for 20 min at 37°C and then subjected to three freeze–thaw cycles followed by five cycles of vortexing (30 s). To remove residual DNA, RNA was digested for 6 h at 37°C with 0.1 U/μL RNase-free DNase I in the presence of 1 U/μL anti-RNase (both from Applied Biosystems, Warrington, UK) and stored at −80°C.

RNA (70–400 ng) was reverse transcribed using 0.6 μM (bacterial 16S rDNA)[13] or 0.8 μM (species-specific ferrodoxin-dependent glutamate synthase[14] and thermostable nuclease gene[15]) primers. RNA from a log-phase culture of *S. aureus* 10850 and the reaction mixture without RNA were used as positive and negative controls, respectively.

Real-time PCRs were carried out in a total volume of 25 μL containing 0.2 μM primers (the ones used for reverse transcription), 12.5 μL of 2× IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, OR, USA) and 6 μL (10–35 ng of total RNA) of the reverse transcription mixture. The cycling conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 10 s, annealing temperature (60°C for 16S rDNA, 55°C for the glutamate synthase gene or 54°C for the thermostable nuclease gene) for 20 s and 72°C for 20 s. Amplification reactions and melt-curve analysis were performed using the iQ5 iCycler thermal cycler and the iQ5 Optical System Software (both from Bio-Rad).

Resuscitation experiments and PFGE typing

Non-culturable biofilms were detached, washed in saline, centrifuged and resuspended in TSB containing the supplements detailed in Table 1. Bacterial growth was checked for 7 days at 24 h intervals by spreading 100 μL of broth onto TSA plates. Grown colonies were tested for their ability to grow on mannitol salt agar and to produce coagulase. PFGE typing was as described previously.[16]

Results

In vitro biofilm production, stress exposure and culturability assays

To induce the VBNC state, *S. aureus* 10850 biofilms developed on membrane filters were placed on NN agar plates without or with antibiotic (vancomycin or quinupristin/dalfopristin) at concentrations equal to MIC or 4×, 8× or 16× MIC (MICs of vancomycin and quinupristin/dalfopristin were found to be 4 and 2 mg/L, respectively). Each condition was run in triplicate. Biofilms were incubated at 37°C and culturability was tested every 2 days. Lack of growth on nutrient medium was noted after intervals ranging from 10 days (16× MIC of quinupristin/dalfopristin) to 40 days (no antibiotic). The time required to reach non-culturability fell progressively with rising antibiotic concentration from MIC to 16× MIC (vancomycin: 30, 25, 20 and 15 days; quinupristin/dalfopristin: 30, 20, 15 and 10 days).

Non-culturable biofilms were detached from the filters, stained by the live/dead method and examined for viable cells by epifluorescence microscopy. All culture-negative filters contained green coccoid cells irrespective of antibiotic type and concentration, with the highest number being found after vancomycin exposure; their number fell with rising antibiotic concentration (Figure 1). In similar experiments where filters were exposed to the same antibiotic concentrations in the presence of nutrients (i.e. rich medium), culturability was preserved for up to 60 days.

Persistence of the VBNC state and gene expression assay

Filters carrying *S. aureus* 10850 biofilms were placed on NN agar plates unsupplemented or supplemented with vancomycin (MIC) or quinupristin/dalfopristin (MIC or 4× MIC). All became non-culturable after 20–40 days.

The number of viable cells was monitored by epifluorescence microscopy for 5 months (Figure 2). In biofilms maintained on antibiotic-supplemented NN agar plates, the number of viable cells was substantially unchanged throughout the experiment (10^3–10^6 cells/mL, accounting for 0.01%–2.3% of the total population), whereas in those maintained on NN agar without

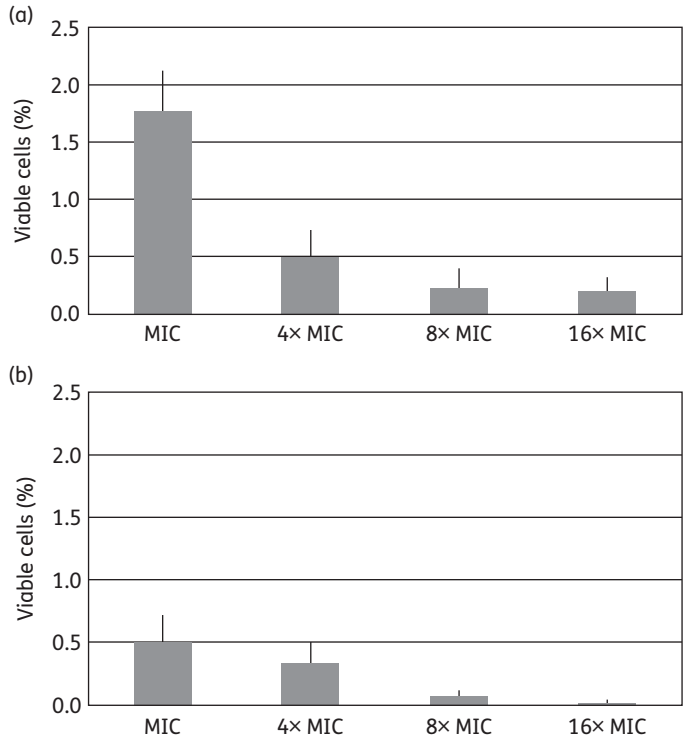

Figure 1. Viable *S. aureus* 10850 cells (%) detected in non-culturable biofilms maintained in NN agar plates supplemented with different concentrations of vancomycin (a) or quinupristin/dalfopristin (b). Cells from 1 mL of the resuspended biofilm were live/dead stained and counted by epifluorescence microscopy. Total counts ranged from 2×10^7 to 3.5×10^8 cells/mL. All values are the mean ± SD of three experiments.

antibiotic, it did not change significantly over the first 90 days and then plummeted.

The presence of viable subpopulations was also investigated by examining gene expression. Aliquots of non-culturable biofilms maintained on NN agar supplemented with vancomycin (MIC) or quinupristin/dalfopristin (MIC or 4× MIC), detached at different times (0, 30, 90 and 150 days) from loss of culturability, were used in real-time RT–PCR experiments targeting 16S rDNA and two *S. aureus* species-specific genes encoding, respectively, glutamate synthase and the thermostable nuclease. Amplicons of the expected size obtained in all assays confirmed the presence of metabolic activity in non-culturable biofilms for ≥150 days.

Epifluorescence microscopy and flow cytometry

Biofilms from NN agar plates without antibiotic or with quinupristin/dalfopristin (MIC or 4× MIC) were detached and examined by flow cytometry 7, 30 and 60 days from loss of culturability. All samples contained three different cell populations: viable, dead and damaged (Figure 3). Viable cells ranged from 10^4/mL to 10^5/mL; comparison with epifluorescence counts demonstrated differences of ≤0.5 log.

Resuscitation experiments and PFGE typing

Resuscitation assays were performed at different intervals from loss of culturability in four different experimental conditions (Table 1). Recovery of culturability was monitored for 7 days at 24 h intervals. Visible growth was detected after 72 h of incubation in the presence of 50% culture filtrate or 0.3% sodium pyruvate (Table 1); notably, this happened only with biofilms that had reached the non-culturable state ≤30 days previously.

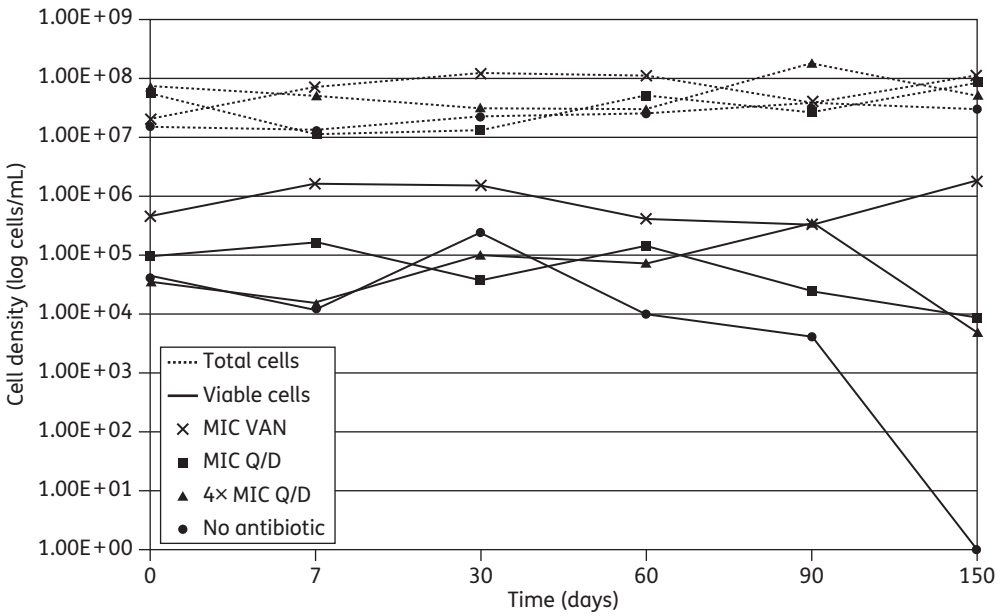

Figure 2. Viable and total cell counts of *S. aureus* 10850 biofilms at different intervals (0–150 days) from loss of culturability. Biofilms were maintained on NN agar plates supplemented with the MIC of vancomycin (VAN), the MIC and 4× MIC of quinupristin/dalfopristin (Q/D) or no antibiotic.

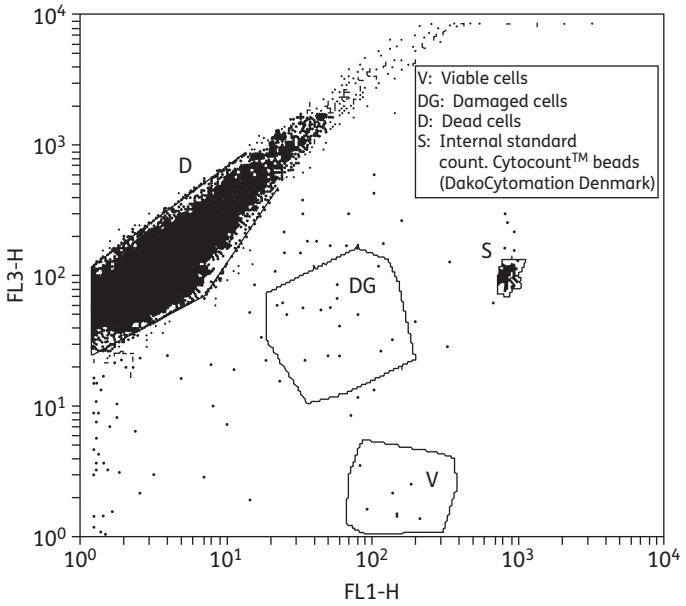

Figure 3. Flow cytometric analysis (FACScalibur flow cytometer; Becton Dickinson, Milan, Italy) of 400 μL of a detached non-culturable *S. aureus* 10850 biofilm (diluted 1:5 in PBS) exposed to the MIC of quinupristin/dalfopristin. FL1-H, channel for green fluorescence (viable cells); FL3-H, channel for red fluorescence (dead cells).

Table 1. Resuscitation of non-culturable *S. aureus* 10850 biofilms detached from filters exposed to quinupristin/dalfopristin (MIC and 4× MIC) 30, 60 or 90 days from loss of culturability

	Time from loss of culturability (days)					
	MIC			4× MIC		
Resuscitation conditions tested	30	60	90	30	60	90
TSB+culture filtrate of *S. aureus* 10850 (50%)	+	−	−	+	−	−
TSB+sodium pyruvate (0.3%)	+	−	−	+	−	−
TSB+glycine (0.15%)	−	ND	ND	−	ND	ND
TSB+glucose (1%)	−	ND	ND	−	ND	ND

ND, not determined.

The identity of the four putatively resuscitated cultures as *S. aureus* 10850 was tested by comparing their SmaI PFGE profiles with the wild-type (WT) strain. Profile differences of a single band proved three cultures (lanes 1, 2 and 3) to be *S. aureus* 10850; the fourth culture (lane 4) showed a completely different electrophoretic pattern and was considered a contamination (Figure 4).

Discussion

Biofilm production hampers antibiotic diffusion and induces an altered phenotype characterized by low metabolic activity, which results in drug tolerance; such conditions promote persistent and recurrent infections.[1] In particular, it has recently been suggested that biofilms carrying VBNC staphylococci might be responsible for CVC-related chronic infections.[10]

This study explores the ability of some stressors mimicking the *in vivo* conditions found in CVC-associated biofilms to induce a true VBNC state in *S. aureus* as well as the ability of the biofilms thus obtained to resuscitate upon exposure to suitable environmental conditions. Exposure of *S. aureus* 10850 biofilms to different antibiotic concentrations showed that non-culturability was achieved in a shorter time in the presence of higher drug concentrations and that the number of VBNC cells markedly declined with the increase in the antibiotic concentration from MIC to 16× MIC. Moreover, no viable cells were detected after 90 days in the absence of antibiotics, whereas in their presence viable cells were detected ≥150 days. These findings suggest that antibiotic exposure in the absence of nutrients can influence the non-culturable state either by contributing to its induction or by promoting its persistence. These data highlight the importance of therapeutic regimens that maintain suitable drug concentrations,[17] especially when treating infections caused by staphylococcal biofilms.

Viable cell counts at different intervals from loss of culturability reflect the role of antibiotic exposure in the survival of biofilm-associated bacterial populations. Indeed, the number of viable cells remained almost unchanged over the 150 days of the study in antibiotic-exposed non-culturable biofilms, but it plummeted after 90 days in non-exposed ones. This behaviour resembles more closely a pre-mortem state or the long-term

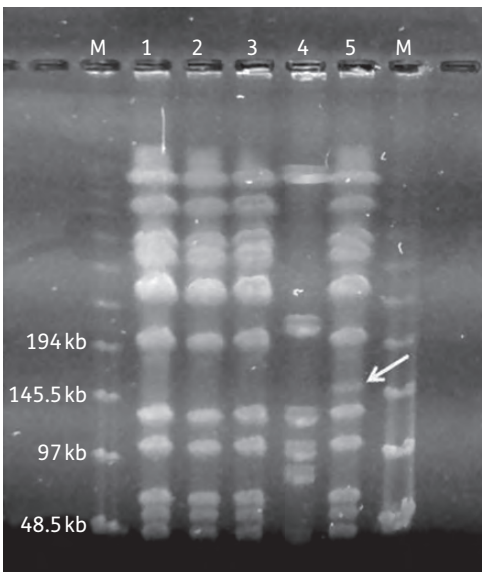

Figure 4. PFGE profile of SmaI-digested total DNA extracted from cultures of putatively resuscitated *S. aureus* 10850 VBNC biofilms and the WT strain. Lanes 1, 2 and 4: biofilms maintained on 4× MIC of quinupristin/dalfopristin resuscitated in the presence of 50% culture filtrate 30 days (lane 1) or 60 days (lane 4) from loss of culturability, or in the presence of 0.3% sodium pyruvate after 30 days (lane 2); lane 3: biofilm maintained on quinupristin/dalfopristin (MIC) and resuscitated in the presence of 50% culture filtrate after 30 days; and lane 5: WT *S. aureus* 10850. M, low-range PFGE marker (New England Biolabs, Ipswich, MA, USA). The arrow indicates the SmaI-deleted band.

survival strategy described in *Enterococcus faecalis* in the aquatic environment,[18] rather than a true VBNC state.

The role of antibiotic stress in VBNC state induction can be explained in various ways. Several authors have reported that the presence of H_2O_2[6,19,20] and increased endogenous oxidative stress[9] may play a significant role in the acquisition of the VBNC state. The inhibition of protein synthesis promoted by quinupristin/dalfopristin could thus contribute to VBNC induction and persistence as a consequence of the failed production of antioxidants, such as catalase (a major feature of staphylococci) and superoxide dismutase, as already reported by others.[19] On the other hand, cell wall modifications caused by vancomycin exposure[21] could result in increased cell wall thickness, a typical VBNC feature.[22] Moreover, since antibiotics can act as regulators of gene expression,[23] they can inadvertently induce cell modifications leading to the VBNC state.

The presence of VBNC *S. aureus* in non-culturable biofilms was confirmed by RT–PCR experiments that documented 16S bacterial rDNA and species-specific gene expression. The quantification of 16S rRNA has previously been reported as a reliable viability assay[24] and since the half-life of bacterial mRNA is typically 3–5 min,[6] continued gene expression by non-culturable cells is considered as an excellent indicator of bacterial cell viability.[25]

In our study, culturability was restored by growth in TSB supplemented with either sodium pyruvate (a peroxide-degrading compound) or culture filtrate of the WT strain (as an exogenous source of resuscitation-promoting or bacterial growth-stimulating factors), as described previously.[8,20] The correspondence with *S. aureus* 10850 of three cultures recovered after resuscitation was borne out by single-band differences in their PFGE profiles, indicating the loss by VBNC cells of a fragment of genomic DNA not essential for survival, a feature that has been related to the VBNC state.[8,26]

In conclusion, we provide evidence that biofilm-embedded *S. aureus* can enter the VBNC state and that antibiotics can be involved in such state transformation and/or its persistence. Since staphylococcal biofilms have been found to cause a variety of persistent infections, the factors promoting VBNC induction and/or persistence (including antibiotic regimens) and resuscitation need to be further explored.

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Transparency declarations

None to declare.

References

1 Agarwal A, Singh KP, Jain A. Medical significance and management of staphylococcal biofilm. *FEMS Immunol Med Microbiol* 2010; **58**: 147–60.

2 Arciola CR, Baldassarri L, Montanaro L. Presence of *icaA* and *icaD* genes and slime production in a collection of staphylococcal strains from catheter-associated infections. *J Clin Microbiol* 2001; **39**: 2151–6.

3 Arciola CR, Campoccia D, Speziale P *et al*. Biofilm formation in *Staphylococcus* implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. *Biomaterials* 2012; **33**: 35967–82.

4 Marshall KC. Nonculturable microorganisms in biofilms. In: Colwell RR, Grimes DJ, eds. *Nonculturable Microorganisms in the Environment*. Washington, DC: ASM Press, 2000; 87–112.

5 Dworkin J, Shah IM. Exit from dormancy in microbial organisms. *Nat Rev Microbiol* 2010; **8**: 890–6.

6 Oliver JD. Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol Rev* 2009; **34**: 415–25.

7 Kana BD, Gordhan BG, Downing KJ *et al*. The resuscitation-promoting factors of *Mycobacterium tuberculosis* are required for virulence and resuscitation from dormancy but are collectively dispensable for growth *in vitro*. *Mol Microbiol* 2008; **67**: 672–84.

8 Trevors JT. Viable but nonculturable (VBNC) bacteria: gene expression in planktonic and biofilm cells. *J Microbiol Methods* 2011; **86**: 266–73.

9 Masmoudi S, Denis M, Maalej S. Inactivation of the gene *katA* or *sodA* affects the transient entry into the viable but nonculturable response of *Staphylococcus aureus* in natural sea water at low temperature. *Mar Pollut Bull* 2010; **60**: 2209–14.

10 Zandri G, Pasquaroli S, Vignaroli C *et al*. Detection of viable but non-culturable staphylococci in biofilms from central venous catheters negative on standard microbiological assays. *Clin Microbiol Infect* 2012; **18**: 259–61.

11 Donelli G, Francolini I, Romoli D *et al*. Synergistic activity of dispersin B and cefamandole nafate in inhibition of staphylococcal biofilm growth on polyurethanes. *Antimicrob Agents Chemother* 2007; **51**: 2733–40.

12 Pianetti A, Manti A, Boi P *et al*. Determination of viability of *Aeromonas hydrophila* in increasing concentrations of sodium chloride at different temperatures by flow cytometry and plate count technique. *Int J Food Microbiol* 2008; **127**: 252–60.

13 Warwick S, Wilks M, Hennessy E *et al*. Use of quantitative 16S ribosomal DNA detection for diagnosis of central vascular catheter-associated bacterial infection. *J Clin Microbiol* 2004; **42**: 1402–8.

14 Martineau F, Picard FJ, Roy PH *et al*. Species-specific and ubiquitous-DNA-based assays for rapid identification of *Staphylococcus aureus*. *J Clin Microbiol* 1998; **36**: 618–23.

15 Depardieu F, Perichon B, Courvalin P. Detection of the *van* alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. *J Clin Microbiol* 2004; **42**: 5857–60.

16 de Lencastre H, Couto I, Santos I *et al*. Methicillin-resistant *Staphylococcus aureus* disease in a Portuguese hospital: characterization of clonal types by a combination of DNA typing methods. *Eur J Clin Microbiol Infect Dis* 1994; **13**: 64–73.

17 Sakoulas G, Moise-Broder PA, Schentag J *et al*. Relationship of MIC and bactericidal activity to efficacy of vancomycin for treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. *J Clin Microbiol* 2004; **42**: 2398–402.

18 Heim S, Lleò MM, Bonato B *et al*. The viable but nonculturable state and starvation are different stress responses of *Enterococcus faecalis*, as determined by proteome analysis. *J Bacteriol* 2002; **184**: 6739–45.

19 Kong IS, Bates TC, Hülsmann A *et al*. Role of catalase and *oxyR* in the viable but nonculturable state of *Vibrio vulnificus*. *FEMS Microbiol Ecol* 2004; **50**: 133–42.

20 Mizunoe Y, Wai SN, Takade A *et al*. Restoration of culturability of starvation-stressed and low-temperature-stressed *Escherichia coli* O157 cells by using H_2O_2-degrading compounds. *Arch Microbiol* 1999; **172**: 63–7.

21 Biavasco F, Vignaroli C, Varaldo PE. Glycopeptide resistance in coagulase-negative staphylococci. *Eur J Clin Microbiol Infect Dis* 2000; **19**: 403–17.

22 Signoretto C, Lleò MM, Canepari P. Modification of the peptidoglycan of *Escherichia coli* in the viable but nonculturable state. *Curr Microbiol* 2002; **44**: 125–31.

23 Modest B, Marahiel MA, Pschorn W *et al*. Peptide antibiotics and sporulation: induction of sporulation in asporogenous and peptide-negative mutants of *Bacillus brevis*. *J Gen Microbiol* 1984; **130**: 747–55.

24 Lahtinen SJ, Ahokoski H, Reinikainen JP *et al*. Degradation of 16S rRNA and attributes of viability of viable but nonculturable probiotic bacteria. *Lett Appl Microbiol* 2008; **46**: 693–8.

25 Lleò MM, Bonato B, Tafi MC *et al*. Resuscitation rate in different enterococcal species in the viable but nonculturable state. *J Appl Microbiol* 2001; **91**: 1095–102.

26 Warner JM, Oliver JD. Randomly amplified polymorphic DNA analysis of starved and viable but nonculturable *Vibrio vulnificus* cells. *Appl Environ Microbiol* 1998; **64**: 3025–8.