MINIREVIEW

Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques

Andreas Nocker[1] & Anne K. Camper[1,2]

[1]Center for Biofilm Engineering, Montana State University, Bozeman, MT, USA; and [2]Department of Civil Engineering, Montana State University, Bozeman, MT, USA

Correspondence: Andreas Nocker, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717, USA. Tel.: +1 406 994 4906; fax: +1 406 994 6098; e-mail: andreas.nocker@gmail.com

Received 28 August 2008; accepted 23 October 2008.
First published online 27 November 2008.

DOI:10.1111/j.1574-6968.2008.01429.x

Editor: Rustam Aminov

Keywords
molecular detection; viability; live–dead distinction; molecular diagnostics; propidium monoazide; nucleic acid amplification.

Abstract

This article elaborates on possible future directions for microbial viability assessment using nucleic acid-modifying compounds in combination with DNA- (and potentially RNA-) amplification technologies. Bacteria were traditionally considered viable when they could be cultured, whereas today's viability concept is based on the presence of some form of metabolic activity, responsiveness, RNA transcripts that tend to degrade rapidly after cell death, or of an intact membrane. The latter criterion was the focus of recent approaches to limit detection to intact cells using ethidium monoazide or propidium monoazide. Membrane integrity must, however, be considered as a very conservative criterion for microbial viability. The new concept presented here aims at limiting nucleic acid-based detection to cells with an active metabolism, which might be a more appropriate viability criterion. To selectively detect only cells with metabolic and respiratory activity (while excluding inactive dead cells from detection), we suggest the use of 'activity-labile compounds'. In addition to their potential usefulness for viability assessment, these new compounds could also be beneficial for selectively amplifying nucleic acids of cells that have metabolic activities of interest. This preferential detection of microorganisms with certain metabolic capabilities is referred to as 'molecular enrichment' in distinction to 'growth enrichment'.

Introduction: the challenge of detecting viable organisms

The ideal scenario in most applications of microbial diagnostics is that only viable cells are detected. In the case of pathogens, only viable cells are likely to pose a threat to humans, animals, or plants. The term 'viability' has been subject to many discussions and is typically referred to as the ability of cells to replicate. As this ability of microorganisms can normally not be determined directly in their natural environment, different techniques to assess viability are used, including traditional cultivation on artificial substrates, detection of RNA, detection of metabolic activity or substrate responsiveness, and the determination of membrane integrity (Keer & Birch, 2003).

This article outlines possible approaches to detect 'viable' cells in combination with nucleic acid amplification methods based on the presence of biological activity. Nucleic acid amplification has become a key technology in microbiology research laboratories and the microbial diagnostic industry due to its versatility, speed, and ability to be automated. In the case of DNA-based methods, however, the indiscriminate detection of cells independent of their viability status poses a severe challenge. This is because DNA can persist for long periods of time in the environment after cells have lost viability (Josephson *et al.*, 1993; Masters *et al.*, 1994). A possible solution is to target RNA, as RNA tends to degrade relatively rapidly after cell death. However, the abundance and stability of RNA in a cell is heterogeneous and there are some RNA molecules that can also persist in cells for extended time periods after loss of viability, depending on the environmental conditions, the method of cell death, the nature and stability of the RNA, and the region that is amplified (Birch *et al.*, 2001). Although not as pronounced as with DNA, this persistence of RNA can, for example, lead to false positive results in the first hours after cell death when monitoring disinfection efficacy (Birch *et al.*, 2001).

The concept of ethidium monoazide (EMA)- and propidium monoazide (PMA)-PCR

In the case of DNA-based detection of bacteria, an important step toward limiting diagnostics to live cells was taken by Nogva *et al.* (2003) by introducing the concept of EMA-PCR. In this approach, viability is based on membrane integrity. EMA is a DNA-intercalating dye with an azide group attached to it. EMA was suggested to enter only membrane-compromised cells (considered 'dead'), whereas the intact membranes of 'live' cells would pose a barrier for the molecule. Once inside membrane-compromised cells, EMA intercalates into the DNA. Such-treated samples are subsequently exposed to bright visible light resulting in covalent binding of the chemical to DNA (photolysis of EMA converts the azide group into a highly reactive nitrene radical that can form a covalent link to DNA). The unbound EMA, which remains free in solution, is simultaneously inactivated by reacting with water molecules. The EMA treatment is followed by extraction of genomic DNA and its analysis by quantitative PCR (qPCR). The covalent crosslinkage of EMA to DNA has been shown to result in a strong inhibition of PCR amplification of the modified DNA. The result of treatment is that only unmodified DNA from intact cells whose DNA was not crosslinked with EMA can be amplified, whereas PCR amplification of modified DNA from membrane-compromised cells is efficiently suppressed. Treatment was thus suggested to lead to the exclusion of cells with damaged membranes from analysis. EMA treatment in conjunction with qPCR led to signal reduction of up to 4 $\log_{10}$ units in the case of membrane-compromised cells (Rudi *et al.*, 2005a, b).

Later studies demonstrated, however, that EMA may penetrate cells with intact membranes (Nocker *et al.*, 2006; Flekna *et al.*, 2007; Cawthorn & Witthuhn, 2008). The extent of EMA uptake by intact cells greatly depends on the bacterial species (Nocker *et al.*, 2006). This problem poses a severe limitation of the use of EMA. Consequently, an evaluation of PMA was performed as a substitute of EMA (Nocker *et al.*, 2006). The comparative study showed that PMA, in contrast to EMA, is efficiently excluded from cells with intact cell membranes, probably due to an increased positive charge. It is applicable to a wide range of gram-negative and gram-positive bacteria. PMA in combination with qPCR monitored killing efficacy by disinfectants (chlorine, ethanol, isopropanol, and benzalkonium chloride) or heat, which share a common mechanism of inducing membrane damage (Nocker *et al.*, 2007a). PMA treatment was also successfully used in molecular ecological studies to limit the detection to intact microbial cells using end-point PCR in combination with denaturing gradient gel electrophoresis (Nocker *et al.*, 2007b). Although further validation is needed, the PMA-PCR method has received positive evaluation in two independent publications, one using *Listeria monocytogenes* and one testing it with pathogenic fungi in air and water samples (Pan & Breidt, 2007; Vesper *et al.*, 2008).

Limitations of PMA-PCR

Problems with PMA-PCR have been encountered with samples from an anaerobic sludge reactor, probably caused by insufficient light transparency due to the presence of black particles (Wagner *et al.*, 2008). This problem of insufficient light-transparency might be solved in the future, using a trigger other than light for inducing the crosslinking event, such as a change in pH or temperature. A more fundamental drawback, however, is that the principle is based on membrane integrity as a viability criterion. Although the efficient exclusion of membrane-compromised cells from the analysis is of benefit, the method does not allow monitoring of the killing efficacy by UV treatment (Nocker *et al.*, 2007a) and other inactivation mechanisms that do not directly target the cell membrane. Membrane damage can in these cases occur as a secondary effect, but little is known about the time span in which membranes disintegrate, which would make these cells susceptible to dye uptake. In an experiment studying post-UV uptake of PMA, the membranes of UV-killed *Escherichia coli* O157:H7 cells did not deteriorate faster than the membranes of non-UV-treated cells when exposed to tap water with a residual chlorine content of 0.5 mg L^{-1} over 120 h in a flow-through system (Nocker *et al.*, 2007a). When subjected to a temperature stress (58 °C) for 5 h instead of residual chlorine, the difference in C_t values between UV-killed and non-UV-exposed cells was only moderate. Our observations correlate with the results by Villarino *et al.* (2000), who showed with *E. coli* K12 cells that were subjected to a lethal UV dose that cell lysis does not immediately follow a loss of culturability. Membrane disintegration might represent the most extreme form of death on the scale between live and dead, suggesting that a more sophisticated live–dead distinction method must account in some way for cellular activity as a viability parameter.

Viability criteria

Different physiological states between 'live' and 'dead' are postulated. Figure 1 shows four of these states in a simplified concept, which is the basis for this article. This model is closely linked to the one described by Nebe-von Caron & Badley (1995). The authors are aware of the wide spectrum of opinions with respect to the definition of viability, but this simple 'working model' was chosen as a practical beginning for the discussion presented herein. Three viability criteria are considered: culturability, metabolic activity,

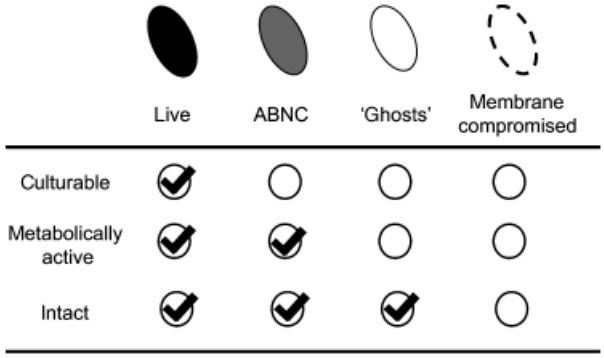

Fig. 1. Schematic representation of selected viability states and their main characteristics relevant for this manuscript.

and membrane integrity. Metabolic activity as a viability criterion is often measured by hydrolysis of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) or reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride. Conversion of these substances or similar chemicals indicates respiratory activity. Culturable cells would fulfill all three viability criteria in this simplified scheme. Cells that are active and intact but cannot be cultured on a standard medium are referred to as 'active but nonculturable' (ABNC) here, whereas intact cells that no longer show detectable respiration and metabolic activity are referred to as 'ghosts'. Membrane-compromised cells would not fulfill any of these three viability characteristics.

Figure 2 schematically represents a model microbial community containing cells in all four of these viability states. When targeting genomic DNA extracted from such a microbial mixture, PCR and isothermal DNA amplification methods amplify DNA from all cells whose genomes are intact in the targeted region. This implies that all cells are detected irrespective of their viability status (Fig. 2a). Sample treatment with PMA, on the other hand, would result in the exclusion of membrane-compromised cells from detection (Fig. 2b). For most applications, it would be desirable to additionally exclude metabolically inactive cells (Fig. 2c). Although dead cells can maintain a residual level of metabolic and respiratory activity (Villarino *et al.*, 2000), there are indications that activity might in many cases be lost faster than membrane integrity. This was observed when studying *Aeromonas hydrophila* survival in seawater microcosms (Maalej *et al.*, 2004). Respiratory activity (measured by CTC reduction) was also lost faster than membrane integrity (measured by uptake of propidium iodide) on exposing *Pseudomonas aeruginosa* biofilms to silver or tobramycin (Kim *et al.*, 2008). With chlorine, on the other hand, the two viability indices decreased approximately at the same rate. It was hypothesized that chlorine can react with inner components of the cell as well as with the cell membrane, whereas silver and tobramycin were more likely to react with the inner components than with the cell membrane after intracellular uptake. Other examples in the literature show the difficulty in making general statements about the correlations between different viability parameters. Despite this heterogeneity and the different rates with which activity and membrane integrity deteriorate dependent on the causative agent of cell death, a molecular viability assay based on biological activity would complement PMA-PCR well. The measurement of more than just membrane integrity in combination with nucleic acid amplification techniques would help to better understand the complexity of cell death.

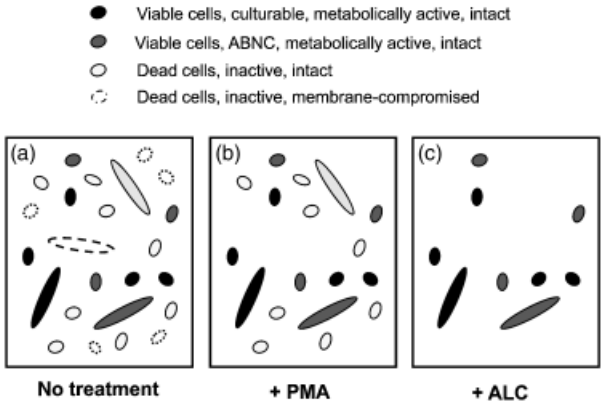

Fig 2. Schematic representation of cells detected (a) without sample treatment, (b) after sample treatment with PMA and (c) ALC.

Concept of activity-labile compounds

This goal could potentially be achieved by sample treatment with 'activity-labile nucleic acid-modifying compounds', abbreviated to 'activity-labile compounds' (ALCs). The conceptual structure of ALCs is compared with the structure of crosslinkable nucleic acid stains (such as PMA) in Fig. 3. Both classes of molecules consist of two components: a

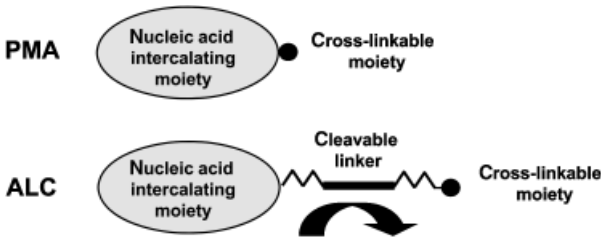

Fig. 3. Comparison of conceptual structures of membrane-impermeant crosslinkable nucleic acids stains (exemplified by PMA) and a representative ALC. In the case of ALCs, the nucleic acid-intercalating moiety is connected with a crosslinkable moiety (e.g. a photoactivatable azide group) via a linker that can be cleaved in the presence of enzymatic or any biological activity. Cleavage results in separation of the DNA-intercalating moiety from the azide group, and thus renders the molecule inactive as it can no longer be crosslinked to the nucleic acid.

DNA-intercalating moiety and a crosslinkable moiety. In contrast to PMA, in the ALC the two components are connected through a linker that can be cleaved by enzymatic activity or any biological activity that results in cleavage. Esterases might be a good example of omnipresent enzymes that are typically found in cells with an active metabolism. Another difference between ALCs and PMA is that ALCs are membrane permeant and thus would enter all cells independent of their membrane integrity. Excess ALCs that do not enter cells are removed by filtration or centrifugation/resuspension of cells. Once inside the cell, two scenarios are conceivable:

(1) In active cells, enzymatic or biological activity results in cleavage of the linker and thus in removal of the azide group from the DNA-intercalating moiety. The latter will intercalate into DNA (or remain intercalated), but due to the absence of the azide group, it can no longer be covalently crosslinked to DNA upon light exposure. In other words, the ALC is rendered nonfunctional in active cells, preventing DNA modification.

(2) In inactive cells, the ALC is not cleaved due to the absence of the activity required for cleavage. In other words, the crosslinkable moiety does not get removed, and the ALC remains functional. Upon intercalation and photoexposure, the ALC is covalently crosslinked to the DNA. This should result in the modification of DNA from inactive cells and thus in their exclusion from the analysis as the modification strongly suppresses its amplification.

The main characteristics of PMA and ALC are summarized in Table 1. The design of different ALC molecules requires optimization of both the DNA-intercalating moiety (e.g. SYTO derivatives; SYTO dyes are commonly used DNA-intercalating dyes) and the crosslinking moiety (e.g. one single azide group or an 'anchor' composed of multiple azide groups to improve the efficiency of covalent linkage). Another parameter to be optimized is the length and type of the intermediate linker. Different combinations of these modules can be synthesized dependent on the application, and the molecules can be tested for their efficiency in reducing detection signals from inactive cells. Regarding the cleavable linker, enzymatically labile ester and peptide bond linkers could be the first linkers to be tested with the reasoning that these bonds do not carry a charge and that the enzymes responsible for cleavage are an abundant group that function with broad substrate specificity. Proof-of-principle for esterases has already been provided by the successful use of microscopic activity stains such as calcein AM and fluorescein diacetate. These nonfluorescent molecules are neutral or near-neutral and can diffuse freely into cells. In the case of metabolically active cells, they are converted by nonspecific cytosolic esterases, which cleave one or multiple ester bonds. The enzymatic conversion takes from 30 min to several hours and produces polar products through the formation of negatively charged carboxyl groups that are retained in the cells. The cleaved products are fluorescent, enabling visualization of active cells with a fluorescence microscope. In inactive cells without esterase activity, which are not capable of conversion, the substrates are not processed and therefore do not fluoresce.

Table 1. Comparison of main characteristics of PMA and ALC

PMA	Membrane impermeant
	Excludes membrane-compromised cells from detection
ALC	Membrane permeant
	Excludes cells without an active metabolism from detection

Overcoming different rates of activity

Apart from parameters such as ALC concentrations and incubation times that will require optimization, the most challenging factor to be overcome will be the biological heterogeneity in the rates of metabolic activities between different species and cells of the same species in different environments. This might require optimized protocols for different bacterial groups and sample types. Another complicating factor is that residual metabolic activity can be maintained for some time even after cell death. It therefore might be beneficial not to detect cells with a measurable activity, but to detect cells that possess the capacity of inducing some form of biological activity. Activity could potentially be induced by the addition of low-molecular-weight dissolved organic carbon that can be utilized easily. Activity inducers would ideally be administered in combination with replication inhibitors to avoid a loss of the quantitative character of the subsequent enumeration by qPCR. The principle of this metabolic responsiveness assay would be similar to the microscopic viability assessment of direct viable counts (DVC). The DVC method was first described by Kogure *et al.* (1979, 1984); it uses yeast extract as a nutrient source and nalidixic acid for inhibition of cell division. This antibiotic inhibits DNA replication without affecting other cellular metabolic activities. As cells continue to metabolize nutrients, they become elongated and can be distinguished morphologically from nonresponsive cells. Later, the method could be improved by incubation with a cocktail of antibiotics instead of one inhibitor alone to overcome potential resistances expressed by some species in complex environmental mixtures (Joux & LeBaron, 1997). Substrate responsiveness can be considered a more sensitive indicator of viability than the presence of respiratory activity, as the chance of residual activity after cell death should be greater than the chance of residual responsiveness. Induction of activity before ALC addition might help to minimize the bias of preferential detection of cells that have a higher activity level and cleave the ALCs faster than less

active cells. Induction might be essential for detecting cells in a dormant state. The necessity for further optimization steps will become obvious once experimental data are obtained. Optimization is likely to be necessary for different species, cells in different growth states, detection of injured cells, differences in cellular characteristics, and other biological parameters. Addressing these potential difficulties would represent an important component of the method validation.

Alternative application of ALCs: 'molecular enrichment'

In addition to limiting the detection to active cells or cells with the capacity of an active metabolism, the ALC concept can be used to selectively amplify nucleic acids from cells that express certain metabolic activities resulting in cleavage of covalent bonds. The linker of the ALCs can be chosen in a way that only cells with the particular activity of interest are able to cleave the linker. Consequently, only these cells can inactivate the ALCs, resulting in preferential amplification of their DNA. In other words, in analogy to enrichment by culture, the presence of ALCs creates a selective environment, which leads to molecular enrichment of those organisms that can inactivate the molecules. This approach permits the design of combinatorial ALC libraries, and customers can choose the ALC molecules of choice. To enrich for species with activity A, one adds ALC-A to the mixture of microorganisms. To enrich for species with activity B, one adds ALC-B to the mixture.

Concluding remarks

The ALC approach is a logical continuation of the EMA/PMA concept for live–dead distinction, but reaches far beyond the potential of these compounds. Like EMA or PMA, the ALC concept is compatible with established rapid molecular diagnostic assays and automated sample analysis. Furthermore, ALCs could be used in combination with PMA treatment and PMA could be added to the sample in the last 5 min of dye incubation for more efficient removal of membrane-compromised cells. Although the experimental validation is yet to be delivered, the concept as outlined here or in a modified version might be an interesting future research direction. All variables (the nucleic acid-binding and crosslinking moieties and the cleavable linker) can be adjusted to the experimental needs; the SYTO moiety and the azide group only serve as examples as they are well known. The same concept, which was described for DNA, applies to RNA in combination with RNA amplification methods (such as nucleic acid sequence-based amplification), in case of delayed degradation of RNA after cell death. When targeting RNA, the concept might also have the potential for protozoa, where the DNA has a more compact structure and is partly enveloped by a nuclear membrane. As an alternative to direct amplification, appropriately chosen ALC labels might allow the physical separation of modified nucleic acids from nonmodified nucleic acids by affinity purification using a binding matrix with a high affinity for the attached label. In case the researcher is interested in the modified fraction, subsequent reversible detachment of the label would potentially restore amplifiability of the purified nucleic acids and hybridization capability.

References

Birch L, Dawson CE, Cornett JH & Keer JT (2001) A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. *Lett Appl Microbiol* **33**: 296–301.

Cawthorn DM & Witthuhn RC (2008) Selective PCR detection of viable *Enterobacter sakazakii* cells utilizing propidium monoazide or ethidium bromide monoazide. *J Appl Microbiol* **105**: 1178–1185.

Flekna G, Stefanic P, Wagner M, Smulders FJ, Mozina SS & Hein I (2007) Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. *Res Microbiol* **158**: 405–412.

Josephson KL, Gerba CP & Pepper IL (1993) Polymerase chain reaction detection of nonviable bacterial pathogens. *Appl Environ Microb* **59**: 3513–3515.

Joux F & LeBaron P (1997) Ecological implications of an improved direct viable count method for aquatic bacteria. *Appl Environ Microb* **63**: 3643–3647.

Keer JT & Birch L (2003) Molecular methods for the assessment of bacterial viability. *J Microbiol Meth* **53**: 175–183.

Kim J, Pitts B, Stewart PS, Camper A & Yoon J (2008) Comparison of the antimicrobial effects of chlorine, silver ion, and tobramycin on biofilm. *Antimicrob Agents Ch* **52**: 1446–1453.

Kogure K, Simidu U & Taga N (1979) A tentative direct microscopic method for counting living marine bacteria. *Can J Microbiol* **25**: 415–420.

Kogure K, Simidu U & Taga N (1984) An improved direct viable count method for aquatic bacteria. *Arch Hydrobiol* **102**: 117–122.

Maalej S, Denis M & Dukan S (2004) Temperature and growth-phase effects on *Aeromonas hydrophila* survival in natural seawater microcosms: role of protein synthesis and nucleic acid content on viable but temporarily nonculturable response. *Microbiol* **150**: 181–187.

Masters CI, Shallcross JA & Mackey BM (1994) Effect of stress treatments on the detection of *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* by the polymerase chain reaction. *J Appl Bacteriol* **77**: 73–79.

Nebe-von Caron G & Badley RA (1995) Viability assessment of bacteria in mixed populations using flow cytometry. *J Microsc* **179**: 55–66.

Nocker A, Cheung CY & Camper AK (2006) Comparison of propidium monoazide and ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J Microbiol Meth* **67**: 310–320.

Nocker A, Sossa K & Camper AK (2007a) Molecular monitoring of disinfection efficacy. *J Microbiol Meth* **70**: 252–260.

Nocker A, Sossa P, Burr M & Camper AK (2007b) Use of propidium monoazide for live–dead distinction in microbial ecology. *Appl Environ Microb* **73**: 5111–5117.

Nogva HK, Dromtorp SM, Nissen H & Rudi K (2003) Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5′-nuclease. *PCR BioTechniques* **810**: 804–813.

Pan Y & Breidt F Jr (2007) Enumeration of viable *Listeria monocytogenes* cells by real-time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. *Appl Environ Microb* **73**: 8028–8031.

Rudi K, Moen B, Drømtorp SM & Holck AL (2005a) Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. *Appl Environ Microb* **71**: 1018–1024.

Rudi K, Naterstad K, Drømtorp SM & Holo H (2005b) Detection of viable and dead *Listeria monocytogenes* on gouda-like cheeses by real-time PCR. *Lett Appl Microbiol* **40**: 301–306.

Vesper S, McKinstry C, Hartmann C, Neace M, Yoder S & Vesper A (2008) Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). *J Microbiol Meth* **72**: 180–184.

Villarino A, Bouvet O, Regnault B, Delautre S & Grimont PAD (2000) Cellular activities in ultra-violet killed *Escherichia coli*. *Int J Food Microbiol* **55**: 245–247.

Wagner AO, Malin C, Knapp BA & Illmer P (2008) Removal of free extracellular DNA from environmental samples by ethidium monoazide and propidium monoazide. *Appl Environ Microb* **74**: 2537–2539.