Gated Materials

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Selective, Highly Sensitive, and Rapid Detection of Genomic DNA by Using Gated Materials: *Mycoplasma* Detection**

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In memory of Enrique Pérez-Payá

Progress in bio-molecular chemistry and nanotechnology has recently resulted in the design of biologically inspired systems with innovative bio-related functions. A key issue in this field is the design of new "smart" systems based on nanoscopic structures and a variety of biomolecules which perform unprecedented functions propelling areas, such as bio-engineering, bio-sensing, bio-nanotechnology, and drug delivery, into new directions.[1] In drug delivery, the development of stimuli-responsive nanoscopic hybrid gated materials involving biomolecules as caps with the ability to release entrapped guests upon application of an external stimulus has attracted tremendous attention.[2–6] These devices contain a support that constitutes a reservoir in which certain compounds can be stored and molecules or molecularly appended objects attached on these containers that act as "gates", allowing the controlled release of entrapped molecules at will. Both components have been carefully selected and arranged to achieve a wide range of control functions. Capped materials have been mainly used in drug delivery applications.[7] In contrast, examples of their use in sensing are much less common.[8] For sensing, the carrier system is commonly loaded with an indicator and the capping mechanism is designed in a way that only a target analyte is able to trigger uncapping and the delivery of the cargo.

Among different bio-molecules, DNA sequences are especially attractive for the design of these gated nanosensors.[4,5] Using this approach the design of a chromo- or fluorogenic nanosensor can be envisioned for which the support is capped with a nucleic acid strand, the selective uncapping of which in the presence of the complementary target oligonucleotide leads to the release of the entrapped dye cargo. We believe that this simple system could contribute to the design of very specific and sensitive devices for easy and quick identification of genomic DNA sequences.

As a proof of principle we designed a sensitive nanodevice for direct and rapid detection of *Mycoplasma*. *Mycoplasma* refers to a genome of prokaryotic microorganisms that are parasites of various animals and plants. In addition, these microorganisms are often present in research laboratories as cell-culture contaminants. A study lead by the DSMZ (German Collection of Microorganisms and Cell Cultures) estimated the occurrence of *Mycoplasma* contamination in at least 20–25 % of continuously cultured cells which can induce errors and misinterpretation of research results.[9–11] In this

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Supporting information for this article (details of general techniques, reagents, synthesis of precursors, synthesis and optimization of **S1-O1**, and a full characterization of compounds and solids) is available on the WWW under http://dx.doi.org/10.1002/anie.201302954.

context, prevention or early detection is the strategy currently in use by a large number of research laboratories. The U.S. Food and Drug Administration (FDA) and DSMZ report on several procedures to detect this contamination of which polymerase chain reaction (PCR) analysis is the most widely accepted.[9,10]

Our proposed approach for *Mycoplasma* detection is briefly represented in Figure 1. MCM-41 mesoporous silica nanoparticles (MSN) of approximately 100 nm have been

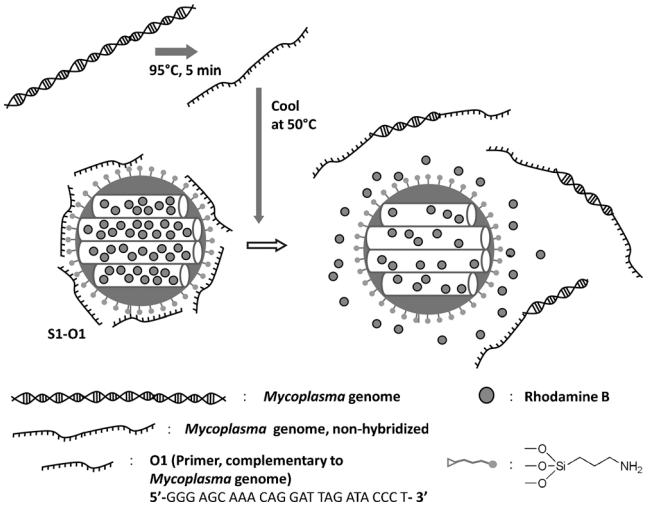

Figure 1. Representation of the gated material **S1-O1** functionalized with APTS and capped with a single-stranded oligonucleotide (**O1**). The delivery of the entrapped guest (rhodamine B) is selectively accomplished in the presence of the complementary non-hybridized 16S ribosomal RNA strand.

selected as the inorganic support because of their unique and advantageous structural properties, such as high surface area (ca. $1000\,m^2g^{-1}$), pore volume ($>1\,cm^3g^{-1}$), stable mesostructure, and tuneable pore diameter (2–10 nm) and morphology. The MCM-41 support was first loaded with a suitable dye (rhodamine B), and then the external surface was functionalized with 3-aminopropyltriethoxysilane (APTS) to give solid **S1**. Aminopropyl groups are partially positively charged at neutral pH in water and could interact with negatively charged oligonucleotides, resulting in the closing of the mesopores. The opening is expected to occur in the presence of a target complementary strand.

As the capping oligonucleotide, a sequence highly conserved in the *Mycoplasma* genome that corresponds to a fragment of the 16S ribosomal RNA subunit was employed (**O1**, 5′-GGG AGC AAA CAG GAT TAG ATA CCC T-3′).[12,13]

The MCM-41 mesoporous nanoparticles were synthesized according to reported procedures.[4a] The amino-functionalized solid **S1** was prepared by suspending 400 mg of calcined MCM-41 and 0.32 mmol of rhodamine B in acetonitrile and stirring the mixture for 24 h at room temperature to achieve maximum loading of the pores. Subsequently, an excess of APTS was added to the suspension. Finally, the pink solid (**S1**) was collected by filtration, washed with acetonitrile, and dried (see the Supporting Information for details).

For the preparation of **S1-O1**, **S1** was suspended in a mixture of H_2O:DMEM buffer 4:6 v/v (pH 7.2) containing the oligonucleotide **O1** (see the Supporting Information). The final **S1-O1** solid was isolated by centrifugation and washed with DMEM to eliminate residual rhodamine B dye and free oligonucleotide **O1** from the capped **S1-O1** material.

The MCM-41 scaffold and the mesoporous solid **S1** were characterized using standard techniques. The main structural properties obtained from these studies such as particle diameter, BET specific surface area, pore volumes, and pore sizes are listed in Table 1 (see the Supporting Information for additional information). Moreover, the contents of APTS and rhodamine B in **S1** and **S1-O1** were determined by elemental and thermogravimetric analyses, showing values of $0.86\,mmol\,g^{-1}$ of APTS and 0.31 and $0.073\,mmol\,g^{-1}$ of rhodamine B, respectively.

Table 1: Main structural properties calculated from TEM, powder X-ray diffraction, and N_2 sorption analysis of MCM-41 and **S1**.

Sample	Particle Diameter [nm]	S_{BET} [m^2g^{-1}]	Pore Volume[a] [cm^3g^{-1}]	Pore size[a] [nm]	Pore size[b] [nm]
MCM-41	80 ± 6	937	0.75	2.75	3.66
S1	80 ± 6	80	0.26	–	–

[a] BJH model. [b] NLDFT model.

The response of **S1-O1** in the presence of *Mycoplasma* was studied employing the genomic DNA of *Mycoplasma fermentans*, a species of *Mycoplasma* suggested to be associated with the pathogenesis of rheumatoid arthritis in humans.[14] For this purpose, 0.5 mg of **S1-O1** were suspended in 2.0 mL of hybridization buffer. Then two aliquots were taken from this suspension. At the same time a DMEM solution containing genomic double-stranded DNA (dsDNA) of *Mycoplasma fermentans* standard (containing 2×10^3 DNA copies μL^{-1}) were heated at 95 °C for 5 min to dehybridize the dsDNA. After cooling to 50 °C, the solution was added to one of the aliquots whereas a DMEM solution (without DNA) at 50 °C was added to the other aliquot. Both suspensions were kept at 37 °C and at certain time intervals, fractions of both suspensions were taken and centrifuged to remove the solid. Dye delivery into the solution was then measured by the fluorescence of rhodamine at 585 nm (λ_{exc} 555 nm). The delivery profiles of rhodamine B in the presence and absence of DNA of *Mycoplasma fermentans* are shown in Figure 2. In the absence of target DNA solid **S1-O1** is tightly capped and shows a negligible release of rhodamine B (Figure 2, curve a). In contrast, the presence of *Mycoplasma* DNA induces the opening of the pores and the subsequent release of the dye. After 90 min, approximately 80 % of rhodamine B was released (30 % of the initial content of dye in **S1-O1**).

Following a similar approach, dye delivery from **S1-O1** was studied as a function of the amount of the genomic DNA copies of *Mycoplasma fermentans* (Figure 3). A correlation between the concentration of genomic DNA and dye delivered was observed in agreement with uncapping involving a displacement of **O1** from the solid surface by the

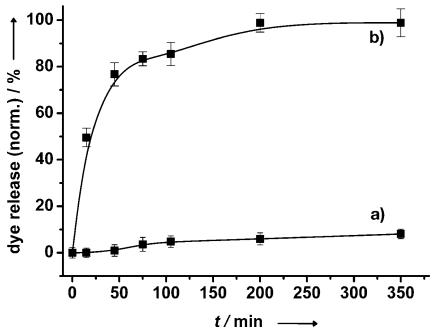

Figure 2. Release of rhodamine B from solid **S1-O1** in the absence (a) and in the presence (b) of genomic DNA of *Mycoplasma fermentans* standard (2×10^3 copies μL^{-1}) in DMEM buffer (pH 7.2).

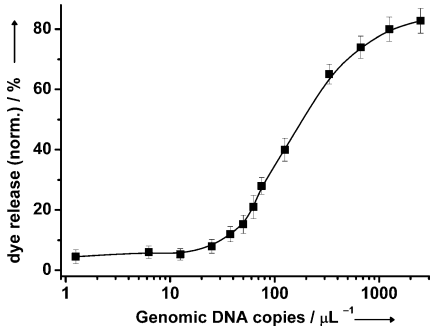

Figure 3. Release of rhodamine B from solid **S1-O1** in presence of different number of genomic DNA *Mycoplasma fermentans* copies in DMEM buffer (pH 7.2).

genomic DNA. The maximum release was observed for concentrations of approximately 900 *Mycoplasma* DNA copies μL^{-1}. The limit of detection (LOD) of this procedure was determined to a concentration as low as 50 DNA copies μL^{-1} (3σ). Figure 4 also shows a photograph of the emission

Figure 4. Emission of rhodamine B (excitation at 254 nm) released from solid **S1-O1** in presence of different number of genomic DNA *Mycoplasma fermentans* copies in DMEM buffer (pH 7.2). From left to right 0, 100, and 266 genomic DNA copies μL^{-1}. Irradiation of the samples was performed using a simple UV lamp.

(excitation at 254 nm) of the rhodamine B released from solid **S1-O1** in the presence of different numbers of genomic DNA *Mycoplasma fermentans* copies (0, 100, and 266 copies μL^{-1}) in DMEM buffer (pH 7.2). A gradual increase in DNA copies induced an enhancement of the emission because of rhodamine B release. A clear emission change is observed between the blank and the samples containing 100 and 266 DNA copies μL^{-1}. On the other hand, for an intermediate point in Figure 3, the number of dye molecules released is around 400-times that of the number of oligonucleotide molecules displaced, which is a remarkable sign of signal amplification.

To verify the selectivity of the method, similar experiments with solid **S1-O1** were carried out in the presence of genomic DNA of the pathogens *Legionella pneumophilia* and *Candida albicans* in amounts of 250 copies μL^{-1}. In addition, and to study the possible interference of other organisms, a mixture containing *Mycoplasma fermentans*, *Legionella pneumophilia*, and *Candida albicans*, each one at a concentration of 250 copies μL^{-1} was also tested. Results are shown in Figure 5. The results show that solid **S1-O1** is highly selective to the presence of genomic DNA of *Mycoplasma fermentans*.

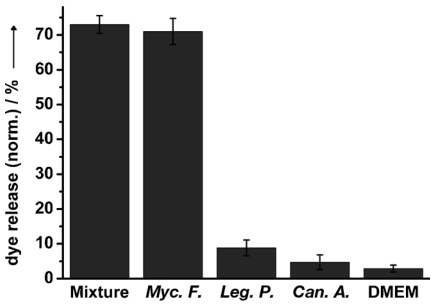

Figure 5. Release of rhodamine B from solid **S1-O1** in the presence of (from right to left): DMEM, DNA genomes (250 copies μL^{-1}) of *Candida albicans (Can.A.)*, *Legionella pneumophilia (Leg.P.)*, and *Mycoplasma fermentans (Myc.F.)*, and a mixture of the DNA of all three pathogens.

Encouraged by these findings we attempted to detect *Mycoplasma* in realistic samples and selected *Mycoplasma*-contaminated cell cultures. In particular, we used the supernatant from two culture media, human cervix adenocarcinoma (HeLa) cells and lung cancer A549 cells, known to be contaminated by *Mycoplasma*, and the supernatant from a culture medium of HeLa cells which was not contaminated. Following a similar procedure to that described above we determined the delivery kinetics of rhodamine B from solid **S1-O1** for *Mycoplasma*-contaminated and non-contaminated cell media (see Figure 6). Despite the presence of a number of proteins and cellular metabolites in the cell-culture media that could have compromised the sensing behavior of **S1-O1**, the results demonstrated that *Mycoplasma*-contaminated samples induced the delivery of the entrapped dye, whereas only a very low release was found for the non-contaminated sample. Moreover, we postulated that the difference in dye released from contaminated HeLa and A549 cells samples was most likely due to the different DNA *Mycoplasma* content in the samples.

As a final step in our research, we were interested to validate the use of this new solid **S1-O1** as a potential alternative to current PCR methods available for *Mycoplasma* detection. Standard PCR techniques were used as a reference method to quantify the contamination of the previously used A549 and HeLa culture media samples with

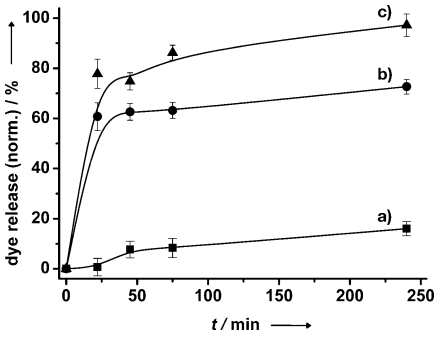

Figure 6. Release of rhodamine B from a) solid **S1-O1** in DMEM culture media of non-contaminated HeLa cells and from culture media of b) HeLa cells and c) A549 cells containing *Mycoplasma* contamination (pH 7.2).

Mycoplasma. For the PCR assay, the sets of oligonucleotides chosen as primers were selected based on their ability to efficiently amplify specific target sequences corresponding to the 16S ribosomal RNA of *Mycoplasma*. The primers selected were **O1** (5′ GGG AGC AAA CAG GAT TAG ATA CCC T 3′) and **O2** (5′ TGC ACC ATC TGT CAC TCT GTT AAC CTC 3′), which allow the amplification of the 280 base pairs (bp) band.[15] The PCR reaction components and cycling were determined through a number of initial trial amplifications based on work by Choppa et al.[12] The PCR results for contaminated A549 and HeLa culture media samples and from *Mycoplasma fermentans* standard solutions at different dilutions are shown in Figure 7. Quantification of the PCR product was based on a comparison between the intensity of the 280 bp band at different dilutions of the contaminated culture samples and of the *Mycoplasma fermentans* standard (using the Multi Gauge V3.2 software). Using this procedure, contents of *Mycoplasma* in the HeLa and A549 culture media of ca. 40000 and 3600 genome copies μL^{-1} were calculated. Moreover, from this study a LOD of approximately 65 *Mycoplasma* DNA genome copies μL^{-1} (3σ) was determined for the PCR analysis.

Figure 7. Agarose gel electrophoresis of PCR-amplified products from different samples showing the presence or absence of the 280 bp band corresponding to *Mycoplasma* genome 16S rRNA fragment. Lines a–e serial dilutions of *Mycoplasma fermentans* standard containing 10^4, 10^3, 10^2, 10, and 1 genome copies μL^{-1} respectively. Lanes f–h correspond to the A549 culture medium in amounts of 100, 10, and 5%, respectively, whereas lanes i–l correspond to the HeLa culture medium in amounts of 100, 10, 1, and 0.5%, respectively. Lane m is water (corresponding to the negative control), lane n corresponds to the DNA ladder and lane o correspond to DMEM medium.

Having established the reference method, the samples were subjected to our newly developed gated-delivery assay employing **S1-O1**. The contaminated A549 and HeLa samples were serially diluted with fresh DMEM to assess performance of **S1-O1** with respect to the limit of detection. Taking the copy numbers as derived from PCR as a reference, these studies revealed a LOD of 70 DNA genome copies μL^{-1} (3σ) for the gated-delivery assay which compares well with the LOD of the PCR analysis (see the Supporting Information). These findings impressively indicate the remarkable performance of the capped system in the fluorometric determination of the contamination of cell culture media with *Mycoplasma*. Moreover as a preliminary study towards possible simple in situ applications we have confirmed that the **S1-O1** material can be stored for weeks and then used without loss of effectiveness.

In summary, we have prepared DNA-capped mesoporous silica nanoparticles loaded with rhodamine B and have used them for the detection of *Mycoplasma* in real contaminated cell-culture media without the use of PCR techniques, allowing a limit of detection of 70 DNA genome copies μL^{-1}. As capping oligonucleotide a sequence highly conserved in the *Mycoplasma* genome corresponding to a fragment of the 16S ribosomal RNA subunit was used. In the presence of the genomic DNA of *Mycoplasma fermentans* an uncapping of the solid occurs inducing the release of the entrapped dye. Reaching similar selectivities, the novel method is easier to handle than PCR, significantly faster (an overall assay time of ca. 60 min compared to ca. 250 min of the PCR method) and has the intrinsic advantage that the signal and not the analyte is amplified, avoiding errors due to miscopying. Moreover the method is much more cost-effective as it only needs a simple UV lamp for the detection of the rhodamine B fluorescence (see Figure 4) versus the more expensive amplification and detection equipment needed in the PCR approach. Although at present the amount of sample that is necessary for the delivery assay is still higher than the amount required for PCR (120 μL vs. 1 μL), this is no limitation for applications such as the present one for which enough sample is available. In conclusion, the coupling of gated-indicator delivery with highly specific biochemical recognition is an innovative strategy for the detection of DNA sequences, able to compete with classical methods which need PCR amplification, in important areas, such as point-of-care diagnostics or detection of specific biological contaminations with pathogens. Such comparatively simple and cheap yet highly selective and sensitive assays hold promise for use in less-developed areas of the world.

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[1] a) R. P. Goodman, I. A. T. Schaap, C. F. Tardin, C. M. Erben, R. M. Berry, C. F. Schmidt, A. J. Turberfield, *Science* **2005**, *310*, 1661 – 1665; b) M. Nishikawa, S. Rattanakiat, Y. Takakura, *Adv. Drug Delivery Rev.* **2010**, *62*, 626 – 632; c) R. Chhabra, J. Sharma, Y. Liu, S. Rinker, H. Yan, *Adv. Drug Delivery Rev.* **2010**, *62*, 617 – 625.

[2] a) A. Schlossbauer, J. Kecht, T. Bein, *Angew. Chem.* **2009**, *121*, 3138 – 3141; *Angew. Chem. Int. Ed.* **2009**, *48*, 3092 – 3095; b) C. Park, H. Kim, S. Kim, C. Kim, *J. Am. Chem. Soc.* **2009**, *131*, 16614 – 16615; c) A. Bernardos, L. Mondragón, E. Aznar, M. D. Marcos, R. Martínez-Máñez, F. Sancenón, J. Soto, J. M. Barat, E. Pérez-Payá, C. Guillem, P. Amorós, *ACS Nano* **2010**, *4*, 6353 – 6368; d) C. Wang, Z. Li, D. Cao, Y.-L. Zhao, J. W. Gaines, O. A. Bozdemir, M. W. Ambrogio, M. Frasconi, Y. Y. Botros, J. I. Zink, J. F. Stoddart, *Angew. Chem.* **2012**, *124*, 5556 – 5561; *Angew. Chem. Int. Ed.* **2012**, *51*, 5460 – 5465; e) C. Coll, A. Bernardos, R. Martínez-Máñez, F. Sancenón, *Acc. Chem. Res.* **2013**, *46*, 339 – 349.

[3] a) Z. Luo, K. Cai, Y. -Hu, L. Zhao, P. Liu, L. Duan, W. Yang, *Angew. Chem.* **2011**, *123*, 666 – 669; *Angew. Chem. Int. Ed.* **2011**, *50*, 640 – 643; b) F. Porta, G. E. M. Lamers, J. I. Zink, A. Kros, *Phys. Chem. Chem. Phys.* **2011**, *13*, 9982 – 9985; c) A. Popat, B. P. Ross, J. Liu, S. Jambhrunkar, F. Kleitz, S. Z. Qiao, *Angew. Chem.* **2012**, *124*, 12654 – 12657; *Angew. Chem. Int. Ed.* **2012**, *51*, 12486 – 12489.

[4] a) E. Climent, R. Martínez-Máñez, F. Sancenón, M. D. Marcos, J. Soto, A. Maquieira, P. Amorós, *Angew. Chem.* **2010**, *122*, 7439 – 7441; *Angew. Chem. Int. Ed.* **2010**, *49*, 7281 – 7283; b) A. Schlossbauer, S. Warncke, P. M. E. Gramlich, J. Kecht, A. Manetto, T. Carell, T. Bein, *Angew. Chem.* **2010**, *122*, 4842 – 4845; *Angew. Chem. Int. Ed.* **2010**, *49*, 4734 – 4737; c) C. L. Zhu, C. H. Lu, X. Y. Song, H. H. Yang, X. R. Wang, *J. Am. Chem. Soc.* **2011**, *133*, 1278 – 1281.

[5] a) V. C. Özalp, T. Schäfer, *Chem. Eur. J.* **2011**, *17*, 9893 – 9896; b) E. Ruiz-Hernández, A. Baeza, M. Vallet-Regi, *ACS Nano* **2011**, *5*, 1259 – 1266; c) Y. Zhang, Q. Yuan, T. Chen, X. Zhang, Y. Chen, W. Tan, *Anal. Chem.* **2012**, *84*, 1956 – 1962; d) D. He, X. He, K. Wang, J. Cao, Y. Zhao, *Adv. Funct. Mater.* **2012**, *22*, 4704 – 4710; e) Z. Chen, Z. Li, Y. Lin, M. Yin, J. Ren, X. Qu, *Chem. Eur. J.* **2013**, *19*, 1778 – 1783.

[6] a) A. Baeza, E. Guisasola, E. Ruiz-Hernandez, M. Vallet-Regi, *Chem. Mater.* **2012**, *24*, 517 – 524; b) D. Tarn, M. Xue, J. I. Zink, *Inorg. Chem.* **2013**, *52*, 2044 – 2049.

[7] a) A. S. Hoffman, *J. Controlled Release* **2008**, *132*, 153 – 163; b) J. L. Vivero-Escoto, I. I. Slowing, B. G. Trewyn, V. S.-Y. Lin, *Small* **2010**, *6*, 1952 – 1967.

[8] a) E. Climent, M. D. Marcos, R. Martínez-Máñez, F. Sancenón, J. Soto, K. Rurack, P. Amorós, *Angew. Chem.* **2009**, *121*, 8671 – 8674; *Angew. Chem. Int. Ed.* **2009**, *48*, 8519 – 8522; b) Y. L. Choi, J. Jaworsky, M. L. Seo, S. J. Lee, J. H. Jung, *J. Mater. Chem.* **2011**, *21*, 7882 – 7885; c) Y. Cui, H. Dong, X. Cai, D. Wang, Y. Li, *ACS Appl. Mater. Interfaces* **2012**, *4*, 3177 – 3183; d) X. He, Y. Zhao, D. He, K. Wang, F. Xu, J. Tang, *Langmuir* **2012**, *28*, 12909 – 12915; e) E. Climent, D. Gröninger, M. Hecht, M. A. Walter, R. Martínez-Máñez, M. G. Weller, F. Sancenón, P. Amorós, K. Rurack, *Chem. Eur. J.* **2013**, *19*, 4117 – 4122.

[9] H. G. Drexler, C. C. Uphoff, *Cytotechnology* **2002**, *39*, 75 – 90.

[10] D. V. Volokhov, L. J. Graham, K. A. Brorson, V. E. Chizhikov, *Mol. Cell. Probes* **2011**, *25*, 69 – 142.

[11] R. S. Barile, *Trends Biotechnol.* **1993**, *11*, 143 – 151.

[12] P. C. Choppa, A. Vojdani, C. Tagle, R. Andrin, L. Magtoto, *Mol. Cell. Probes* **1998**, *12*, 301 – 308.

[13] F. J. M. Van Kuppeveld, T. M. Vanderlogt, F. Angulo, J. Vanzoest, W. G. V. Quint, G. M. Niesters, M. D. Galama, W. J. G. Melchers, *Appl. Environ. Microbiol.* **1992**, 2606 – 2615.

[14] a) T. Sohaeverbeke, C. B. Gilroy, C. Bebear, J. Dehais, D. Taylor-Robinson, *Lancet* **1996**, *347*, 1418 – 1418; b) S. Horowitz, B. Evinson, A. Borer, J. Horowitz, *J. Rheumatol.* **2000**, *27*, 2747 – 2753.

[15] F. J. M. Van Kuppeveld, K. E. Johansson, J. M. D. Galama, J. Kissing, G. Bolske, J. T. M. Van Der Logt, W. J. G. Melchers, *Appl. Environ. Microbiol.* **1994**, *60*, 149 – 152.

