Bacteria quorum sensing in silica matrices

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Serratia marcescens bacteria were encapsulated in silica gels containing glycerol. In agreement with previous studies on Escherichia coli, entrapped cells showed a ca. 50% viability rate after one month. Nutrients were provided to the bacteria, allowing the production of prodigiosin, a red pigment exhibiting some promising therapeutic properties. Addition of "quorum sensing" molecules involved in intercellular communication leads to an enhanced prodigiosin production after four subsequent recyclings of the bacteria-containing gels over one month. Moreover, at the end of this period, nearly 100% of the initial cell population remain viable within the gels. These results suggest that, in the presence of "quorum sensing" molecules, S. marcescens bacteria can enter a stationary state where their metabolism is modified, enhancing their resistance to the stresses induced by encapsulation.

Introduction

The sol-gel encapsulation of biomolecules is becoming a very popular method. Inorganic matrices offer several advantages compared to polymers currently used for bioencapsulation. They exhibit improved mechanical strength and chemical stability and they do not swell in water, preventing the leaching of trapped molecules. Up to now most studies have been performed with metallo-proteins, enzymes or antibodies. More recently whole-cell micro-organisms have also been encapsulated in sol-gel matrices. Bacteria have been shown to retain their activity, opening new possibilities for the realisation of biosensors and bioreactors. However, one of the main challenges for a real use of bioencapsulation in inorganic matrices as a generic method remains the ability to maintain the long-term viability of trapped micro-organisms.

Recent experiments have shown that the sol-gel process could be improved to preserve the viability of trapped Escherichia coli. About half of these bacteria remain viable after one month when sol-gel encapsulation is performed with aqueous precursors, in the presence of glycerol, a well-known cryo-protective agent currently used for bacteria conservation. Because of space limitation, trapped bacteria cannot divide any longer. Thus, during these experiments, nutrients were not provided to encapsulated cells in order to limit their growth propensity. However, they adapt their metabolism to these new conditions and remain culturable, forming colonies again when the gel is redispersed in a culture media. Moreover, they still exhibit glucose uptake and glycolysis activity.

This paper reports on the encapsulation of Serratia marcescens. These bacteria produce a red pigment, called prodigiosin, that exhibits some promising therapeutic properties. In a step forward to the optimisation of bacteria viability and metabolic activity within sol-gel silica matrices, the effect of acylated homoserine lactones as "quorum sensing" (QS) molecules is investigated in Gram-negative bacteria, these molecules are involved in the expression of genes as a function of cell population density. They are specifically released as diffusible signals for cell-to-cell communication within a bacterial population and have been shown to regulate cellular adaptation to changing environmental conditions. They could therefore be helpful to maintain the viability of bacteria encapsulated in sol-gel matrices.

Experimental

Materials

Serratia marcescens (CIP-107492) was provided by the Institut Pasteur collection (Paris, France). Silica sources were sodium silicate solutions SiNa (27 wt% SiO$_2$, 10 wt% NaOH from Riedel-de Haën) and colloidal silica (LUDOX HS-40 from Aldrich). The following growth media and buffers were used: tryptose casein soy broth (TCS), phosphate buffer (NaH$_2$PO$_4$: 34 mM, K$_2$HPO$_4$: 64 mM, (NH$_4$)$_2$SO$_4$: 20 mM, MgSO$_4$: 0.3 mM, pH 7) supplemented with 0.5% w/w casamino acids, 10% w/w glycerol and 5 μM N-butanoyl-l-homoserine lactone (BHL) or N-hexanoyl-l-homoserine lactone (HHL).

Bacterial strain growth conditions

Stock cultures of cells were prepared from cultures stored at –80 °C in TCS broth supplemented with glycerol. When needed, cultures of bacteria in TCS broth at 30 °C were prepared overnight. An inoculum (0.1 ml) was added in a same broth. After 9 hours, an inoculum (0.1 ml) of the culture was added to the phosphate buffer (200 ml) in a flask (1 l) and grown at 30 °C under stirring (200 rpm). After 12 h of incubation, the culture was harvested by centrifugation at 6000 rpm for 15 minutes at 9 °C. The pellet was washed twice with a phosphate buffer (100 mM), and diluted to a concentration of 10$^6$ cells ml$^{-1}$ in a phosphate-glycerol buffer to obtain the working cell suspension (WCS).

Encapsulation of cells

SiNa (0.4 M, 1 ml) was mixed with LUDOX (8.5 M, 1 ml) and hydrochloric acid (4 M) was added until neutralisation. The WCS solution (1 ml) was immediately added and the mixture homogenised under gentle stirring (300 rpm). Gelation

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occurred within about 2.5 min at room temperature. The final bacteria concentration in gels (SG) was close to $3.3 \times 10^6$ cells ml$^{-1}$. Wet gels were aged for 24 hours at 20 $^\circ$C in the mother solution in a closed flask in order to ensure gel densification before analysis.

Xerogels containing cells were obtained by drying at 60 $^\circ$C and characterised using scanning electron microscopy (SEM). Powders were coated with gold in a Balzers Union SCD 40 sputter coater and studied on a Cambridge Stereoscan instrument at an accelerating voltage of 20 kV.

**Biosynthesis and extraction of prodigiosin**

Phosphate glycerol buffer eluants, supplemented or not with BHL or HHL (6 ml) were deposited over SG gels, or added to WCS suspensions supplemented with phosphate–glycerol buffer (2 ml) to obtain the same dilution as in gels. Samples were stored at 20 $^\circ$C in a closed flask. At different times, an aqueous NaCl saturated solution (1 ml) with isopropanol (1 ml) and hydrochloric acid 10 M (0.3 ml) was mixed with aliquots of eluant (1 ml). After vigorous shaking, the mixture was harvested by centrifugation at 2500 rpm for 10 min. The concentration of prodigiosin in the supernatant was determined by measuring the absorbance at $\lambda = 540$ nm, using an UVIKON XS spectrophotometer.

After one week, the supernatant was withdrawn and 6 ml of phosphate–glycerol buffer added on the gel surface. After 30 min, the supernatant was sampled and the amount of remaining prodigiosin checked spectrophotometrically. The washing process was repeated until the supernatant contained no pigment. Fresh eluant solutions were then deposited on the gel surface and prodigiosin production studied for another week. This procedure was repeated twice in order to study the biosynthesis of prodigiosin over four cycles.

**Culturable cell enumeration**

**Bacteria in gel supernatant.** A series of 10-fold dilutions in phosphate buffer of aliquots (0.1 ml) of supernatant solutions were surface-plated in triplicate on TCS-agar. Plates were then incubated at 30 $^\circ$C for 24 h. The colony forming units count before encapsulation is taken as a 100% reference.

**Encapsulated bacteria and WCS.** Wet gels were crushed, vigorously stirred with phosphate buffer (3 ml) for 1 hour after one, two and four weeks. A series of 10-fold dilutions in phosphate buffer of WCS or resuspended gels (0.1 ml) diluted in phosphate buffer were surface-plated in triplicate on TCS-agar. Plates were then incubated at 30 $^\circ$C for 24 h. The colony forming units count of WCS before encapsulation is taken as a 100% reference.

**Results and discussion**

**Effect of encapsulation on S. marcescens viability**

The entrapment of *Serratia marcescens* was performed as previously described.\textsuperscript{15,16} Bacteria taken from their culture medium during the exponential growth phase, were suspended in an aqueous buffer solution containing glycerol (10% w/w). Encapsulation is obtained by adding an aqueous solution of silicate precursors giving a silica gel with few minutes. During these experiments, no nitrogen source was provided in order to limit their growth propensity, which could induce some stress on immobilised cells.

As a first step, it was important to check whether the encapsulation process successfully developed for *E. coli* bacteria could be extended to other cellular strains. Therefore, the viability of bacteria in phosphate buffer solutions supplemented with glycerol (WCS) and within the glycerol-containing silica gel (SG) was studied via the culturable cell enumeration technique. The evolution of colony formation units (CFUs) for both bacteria populations is shown on Fig. 1. Upon ageing, the amount of CFUs decreases in all samples. It appears that, after one month, more bacteria remain culturable in the gel (45%) than in the aqueous suspension (5%). These results are similar to those obtained with *E. coli*,\textsuperscript{15,16} suggesting that the glycerol–silica matrices may be suitable hosts for a large variety of bacteria.

Additionally, SEM studies revealed that imprints left by bacteria destroyed during the sample preparation appear homogeneously distributed over the gel surface (Fig. 2a) while their size (ca. 2 $\mu$m $\times$ 0.5 $\mu$m) (Fig. 2b) correspond to usual bacteria dimensions.

**Growth of S. marcescens and prodigiosin production**

As glucose was shown to inhibit the formation of prodigiosin,\textsuperscript{28,29} casaminoacids, a mixture of different amino acids, was used as the carbon and nitrogen source in these studies.

![Fig. 2](image2.png) SEM micrographs of bacteria imprints on silica gel surface showing (a) bacteria dispersion and (b) bacteria size.

![Fig. 3](image3.png) Growth curve of *S. marcescens* in a phosphate buffer supplemented with casaminoacids as determined by the optical density at $\lambda = 600$ nm ($\text{OD}_{600}$) over 48 h and corresponding prodigiosin concentration.
Fig. 3 shows the growth curve of *S. marcescens* in a phosphate buffer containing 0.5% w/w casamino acids. The optical density (OD$_{600}$) of bacteria suspensions at $\lambda = 600$ nm, is known to be proportional to the density of cells. Thus, after a short period where bacteria adapt themselves to the new medium, a rapid development phase is observed. A plateau is then obtained after about 20 hours, showing that the bacteria population reaches a stationary growth phase. Also shown on Fig. 3 is the evolution of prodigiosin production with time, as monitored by optical density measurements at $\lambda = 540$ nm. Both curves are closely parallel, in agreement with literature data.$^{29-32}$

**Effect of QS molecules on *S. marcescens* cultivability and prodigiosin production**

**Bacteria suspensions.** Bacteria cultivability and pigment production were studied simultaneously over one week. The production rate for all samples is maximal from the beginning of the experiment, and reaches a plateau after 2 days (Fig. 4a). Additional “quorum-sensing” signalling molecules do not increase the production of prodigiosin.

In contrast to *E. coli*, the amount of culturable bacteria in the solution decreases rapidly with time (Fig. 5a). Indeed, a phosphate buffer was used for the conservation of *E. coli* bacteria, whereas nutrients are added to *S. marcescens* allowing bacteria to finish their growth and to enter in the declining phase of growth. Fig. 5a shows that the number of culturable *S. marcescens* increases after some time, suggesting that QS molecules may influence the growth of bacteria but not the production of metabolites.

Comparing the production of prodigiosin and the growth of bacteria after one day suggests that this pigment is, in fact, synthesised during the first 24 hours following encapsulation.

Further on, residing bacteria in the suspension and even QS induced new cells do not appear to be able to produce noticeable amounts of prodigiosin. This may result from a retro-inhibition process arising when large amounts of prodigiosin are present in the medium.

**Encapsulated bacteria.** Bacteria cultivability in the gel supernatant and pigment production have been studied simultaneously over one week. Prodigiosin production within the gels appears slower than for suspended bacteria (Fig. 4b). Moreover, it seems that the presence of the QS molecules increases the production yield. However, no plateau is observed after one week so that the possibility for the three production curves to reach a common value after a longer time scale should not be put aside. As far as culturable bacteria in the supernatant are concerned (Fig. 5b), the CFU counts remain very low, in the absence or presence of QS molecules.

Observed differences in production kinetics and yield should be attributed to the gel properties. As a matter of fact, prodigiosin must diffuse through the pores of the gel to reach the supernatant solution before it can be detected. This might explain the apparent slower rate of production. However, this diffusion is not only a drawback. As a matter of fact, it allows the produced prodigiosin to be separated from the entrapped bacteria so that the retro-inhibition effect probably occurring in suspension is more limited here, leading to better production yield. Actually this effect was previously used to develop a prodigiosin bioreactor with an internal adsorbent to increase its production.$^{33}$

**Prodigiosin production recycling**

Fig. 6 shows the evolution of OD$_{540}$ seven days after each of four subsequent recycling steps, in a phosphate buffer
containing glycerol, casaminoacids and no QS molecules, BHL or HHL. In all cases, prodigiosin production decreases rapidly after two uses and then seems to remain constant at a level of ca. 50% of the initial production. However, the presence of the QS molecules enhances the production rate of about 20%.

One possible explanation of such an enhancement could be a longer viability of trapped bacteria in the presence of QS molecules. In order to check this hypothesis, the number of culturable bacteria within the gels after one month was investigated using the plate count technique. Taking the CFU values before encapsulation as a reference, the percentage of culturable bacteria in the absence of QS molecules and in the presence of BHL or HHL were respectively 40%, 60% and 100%, with an error range within 10–20%.

This last value may appear rather surprising but could reflect two different situations. Cell division remains possible in the gel and dead bacteria are replaced by new ones. However, such a growth process implies cell division inside the gels which, according to previous results on E. coli entrapment, should not occur as a result of bacteria confinement. This suggests that only the bacteria population initially entrapped can contribute to prodigiosin production.

Thus, a second possibility should be envisaged which implies that QS molecules maintain the entrapped bacteria in a better physiological state, limiting cellular death. As a matter of fact, it was shown that, in the presence of quorum sensing molecules, bacteria may enter a stationary state where their metabolism is modified, enhancing cells resistance to external stresses.

**Conclusion**

Despite a number of advantages, mainly related to their physico-chemical stability, when compared to polymer systems, sol-gel silica matrices are not yet considered as suitable hosts for cell immobilisation. In fact, when entrapped within polymer hosts, cells are still able to divide and the micro-organism population is continuously renewed. In the case of sol-gel matrices, such division is no longer possible so that efforts should be made to maintain cell viability over a long period of time. This is only possible if the micro-organisms adapt their metabolism to their new confined environment.

Such adaptative processes are widespread in natural systems. Some of them rely on the possibility for a cell population to exchange signalling molecules, as illustrated here by the bacteria “quorum sensing” process. This work suggests that adding such QS molecules significantly improves the viability of *Serratia marcescens* bacteria over one month. As a result, over the same period of time, the production of prodigiosin, a metabolite exhibiting promising therapeutic properties, is noticeably enhanced in the presence of QS molecules.

Nevertheless, several improvements should now be performed to optimise this system. A better oxygenation of the entrapped cells should favour prodigiosin production. Modification of the silica gel surface may be undertaken to control pigment diffusion and facilitate its recovery. Finally, the understanding of QS molecules effect on bacteria behaviour remains a true challenge, not only in the context of cell bio-encapsulation but also, on a more general level, in the fascinating field of cellular communication.

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