# The water clock of Proteus mirabilis paces colony periodic and synchronous swarming

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**For decades, the origin of the concentric ring pattern of bacterial swarming colonies has puzzled microbiologists. Thanks to *in situ* and real time infrared microspectroscopy and the brilliance of the infrared beam at SOLEIL synchrotron, we demonstrate here that *Proteus mirabilis* swarming is paced by a periodic variation of the water activity at colony's edge. This periodic variation originates a phase transition within the extracellular matrix water H bond network which switches on and off the exopolysaccharides viscoelasticity and, consequently, the ability of bacterial cells to swarm. A dynamic behaviour emerges from the global properties of the multicellular entity which here relies on the ability of the bacterial cells to tune exoproducts synthesis in order to undergo sharp transitions above/below a given water activity threshold.**

Bacterial colonies constitute experimental systems of choice for studying fundamental problems of self-organization and pattern formation in complex biological systems. This is the reason why development biologists have turned one's attention to multicellularity[1] since such complex behaviours necessarily imply a high degree of signal integration leading to correlate in space and time the behaviour of billions of individual cells . Hence, the interest of such studies significantly overpasses that of bacterial colonies and biofilms. In the case of *Proteus mirabilis*, it has been proposed that the periodicity of the swarming phenomenon[2-5] was linked to density-dependent thresholds in controlling the transitions between distinct phases[3]. Such correlations, often observed in bacterial multicellular entities, usually rely on chemical triggers, which mediate the quorum sensing (QS) cell-to-cell exchanges[6]. More precisely, QS allows every cell to sense the population extent and, beyond a given density threshold, to activate the genes responsible for differentiation or virulence expression. Regarding this point, the *Proteus mirabilis* swarming constitutes an unique complex biological oscillator. The population expanses through periodic and synchronous swarming phases which alternate with consolidation phases (Fig. S1). If one excepts the very early stages of the colony formation, no chemical trigger like furanones or homoserine lactones are produced to warrant this unique synchronicity[4]. If the flhDC operon has been identified as the main genetic determinant for the vegetative to swarmer cell differentiation process[7], genetics failed to reveal which factor was responsible for the sudden operon's switch at a given moment. Consequently, chemical triggers were actively, but in vain, looked for[6] since they are good candidates to convey information between cells, allowing thereby to locally promote population cohesion[4]. This direction being a dead end, we turned to investigate the global properties of the colony and paid attention to the biochemical and functional properties of the extra cellular matrix (ECM) that constitutes the bacterial cells continuum[8, 9] into which evolves the bacterial population. The *P. mirabilis* ECM biochemical analysis revealed a binary mixture of exopolisaccharides (EPS), a

phenoglycolipid (PGL) and glycine betaine (GB), a well known bacterial osmoprotectant. The presence of GB and of a PGL strongly suggested that the bacterial colony is submitted to a permanent osmotic stress which in turn indicates that water must acts as a limiting factor.

*In vitro* experiments performed on the ECM or on EPS fractions demonstrated a marked viscoelastic behaviour with sharp phase transitions driven either by temperature or water activity (aw)[8]. Such a non-linear behaviour relies on EPS auto organization, which in turn depends on the physico-chemical modulation of polysaccharides H bond networks. A mere 3 times dilution of the EPS fraction relative to its physiological concentration within the colony (300 $g.L^{-1}$) leads to the loss of the viscoelastic property. Additionally, the presence of spherolites has also supported that ECM behaves as a semi crystalline medium[8,9]. Knowing that the external medium viscosity is an acknowledged stimulus to drive the initial cell differentiation subsequent to the transfer of the strain from a liquid culture to an agar Petri dish[10], we have hypothesized that the periodicity and synchronicity of the swarming could be ruled by aw variations at the edge of the colony. Both experimental observations and theoretical considerations[9,11] converge to suggest that, due to the colony spreading on the agar as a thin (15 µm) film during a swarming phase, the resulting increased S/V ratio would increase the net water transfer from the agar to the colony. The resulting increased aw within the biofilm should consequently induce a disorganization of the EPS H bond networks which are responsible for the ECM viscoelasticity. If true, an aw periodic variation at the edge of the colony must be responsible for the swarming periodicity and synchronicity since the latter is linked to the ECM viscosity.

We firstly attempt to assess *in situ* the occurrence of EPS conformational changes by monitoring in a non-invasive way the EPS spectral domain through an entire swarming cycle *i.e*. between two consecutive consolidation phases. Every two minutes, a spectrum

was collected at the colony's moving edge, which allows mapping the spectral information. The Fig. S2 shows the resulting MIR spectra[16]. The data set was analyzed by Principal Component Analysis (PCA) and the first principal component featuring 45% of the total variability was plotted as a function of the colony expansion (Fig. 1A). The resulting data show that most of the spectral variations, can be safely assigned to EPS since the three first PCs, reflecting up to 90% of the total variability, are dominated by spectral features which correspond to the EPS absorption domain (Fig. 1B). It is noteworthy that the PC1 scores clearly undergo reversible sharp transitions as the colony's edge switches from a swarming to a consolidation phase. Hence, it can be assess that the EPS conformational changes which have been observed *in vitro*[8, 17], also occur *in situ* at the transition between consolidation and swarming phases.

In order to determine whether or not these EPS conformational changes are linked to aw variations within the colony, IR spectra were collected on a wider spectral window to encompass both the EPS and water (librations mode) frequency domains. This was made possible by the use of a Si:B bolometer which is much more sensitive in the low frequency domain than the MCT detector. The Fig. S3 shows the mean spectrum and the amplitude variations of the data set collected, as previously, during a full swarming cycle. Besides variations in the EPS spectral domain, significant variations are also observed in the spectral range that corresponds to the water librations mode *i.e*. from 900 to 400 $cm^{-1}$. A PCA and 2D correlation analyses were performed to characterize the link between the variations occurring in these two domains. The EPS conformational changes are again well identified after a PCA performed on the whole spectrum (2000-400 $cm^{-1}$): the spectral patterns of PCs loadings (Fig. 2B) are typical of polysaccharides absorption spectra whereas the symmetric time pattern of the PC1 scores again assesses that these conformational changes are correlated with the swarming/consolidation transition. To emphasize spectral variations of the water librations mode, a PCA was performed in a narrower domain (900-400 $cm^{-1}$). From the

Fig. 2C it clearly appears that the PC1 scores plotted as a function of time exhibit a symmetric pattern as previously observed in Fig. 2A. The corresponding loadings (Fig. 2D) show that during swarming, the water band looses intensity over its lower energy side. This spectral evolution has been reported (see Fig. 2C in 15) to reflect an increased crystallinity or, in other words, a larger H bonds connectivity. To emphasis a correlation between the EPS H bond networks and changes in water amount and connectivity, a 2D correlation analysis was performed[18]. The corresponding synchronous 2D correlation map (Fig. 3A and Fig. S4) indicates that the spectral envelope between 950 and 1050 $cm^{-1}$ decreases as the 500 to 800 $cm^{-1}$ domain increases. Indeed, in a 2D synchronous map, negative cross peaks reflect bands that evolve in an opposite manner during the process[18]. Information about the time sequence of these spectral variations can be derived from 2D asynchronous maps. The Fig. 3B shows a part of the upper left corner of the 2D asynchronous map which can be seen in Fig. S5. The negative cross peak centred at 1000 / 700 $cm^{-1}$ allows to establish that the EPS vibrational bands are altered after the water ones[18, 19]; hence, changes in the water H bond networks at the colony's edge precede any EPS conformational changes.

Knowing that polysaccharides solutions allow forming extensive H bond networks, the marked viscoelastic behaviour of the ECM networks must be linked to the periodic ability of the colony to swarm. In this scheme, consolidation must be triggered by the ECM loss of viscoelasticity. A wide infrared transparency window combined with the brilliance of the IR synchrotron beam at SOLEIL has allowed us for the first time to simultaneously monitor, in a non-invasive way, the water content and the EPS conformational changes within a currently swarming colony. Statistical data analysis (PCA and 2D correlation spectroscopy) have allowed to establish that periodic variations of the aw and the resulting water H-bonding changes do periodically occur and trigger EPS conformational changes. The progressive restriction of free water molecules during a consolidation phase increases the connectivity of the EPS-water H

bond networks and consequently allows recovering the ECM viscoelasticity required to initiate a new swarming phase. Hence, the extensive correlation which links individual cells over large distances originates in a global property of the multicellular entity. The periodic water gradient at the colony's edge associated to geometrical variations of the colony which spreads from a "thick" (> 45 µm) consolidation terrace to a "thin" (16 µm) swarming film warrants for the synchrony of swarming. This well demonstrates how biological properties and physical laws interplay: the bacterial cells synthesize a mixture of exoproducts (EPS, PGL, GB) which structures are tuned to undergo marked transitions as passing over a given threshold. The *P. mirabilis* exoproducts differ whether the strain is grown in a liquid culture or on a solid medium[8]; this further supports that the ability of bacterial populations to cope with distinct environments tightly relies on the behaviour of such macromolecules.

In conclusion, the periodic and synchronous population behaviour is not controlled by a chemical trigger, but rather derives from the potential of supramolecular structures to respond to an external stimulus yielding thereby a high order over large dimensions. These results concerning the *P. mirabilis* biologic oscillator are of broad significance since they demonstrate how the non-linear behaviour of macromolecules promotes complex embedded dynamic cycles which root the evolution from unicellularity to multicellularity and, eventually, to pluricellularity : the permanent osmotic force allows polysaccharides self-organization to promote cyclic dissipative structures.

**Methods.**

The Mid Infrared (MIR) spectroscopy has for long been used to probe macromolecule structures and conformations and, hence, is well suited to study EPS conformational changes[12-14] which show up in the 1200-900 $cm^{-1}$ frequency domain[12, 13]. Water is also a

strong absorbent in the MIR domain due to the O-H stretching (localized at around 3400 $cm^{-1}$) and the H-O-H bending (localized at 1640 $cm^{-1}$) vibrations. Unfortunately, due to significant spectral overlap, essentially with proteins, the colony water content cannot be straightforwardly derived from absorbencies in these domains. This drawback may be bypassed by monitoring instead the water librations band located at around 615 $cm^{-1}$ [13-15]. This band is rarely used since most of the optics and detectors of infrared spectrophotometers are non-transparent and not sensitive enough, respectively, to get a signal with sufficient SNR to be interpreted. Another technical difficulty is that bacterial colonies exhibit high absorbency and diffusivity in the infrared domain which prevent to collect a high quality specular reflected signal, the unique acquisition mode to get spectral information without any physical contact between the optics and the colony. As a consequence, is required to use a very brilliant IR source only available at a synchrotron radiation facility. Hence, to simultaneously monitor the EPS conformational changes and aw variations during a complete swarming phase, we have used synchrotron infrared microspectroscopy (Continuμm and NicPlan, Nicolet) allowing a satisfying (15 μm) 2D spatial resolution, and two types of IR detectors: a MCT detector to firstly assess *in situ* the occurrence of EPS conformational changes and, secondly, a Si:B bolometer which allows to record IR spectra in the 2000-400 $cm^{-1}$ wavenumber range which encompasses both EPS and water librations absorption bands. Spectra are the average of 126 scans and are collected at a 4 $cm^{-1}$ spectral resolution.



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## Figure legends

Fig. 1. EPS undergo periodic conformational changes during a swarming phase as deduced from the symmetric pattern of the PC1 scores (A) and PCs loadings since the three first principal components shown in (B) assess that major EPS conformational changes occur during the swarming phase as deduced from marked peaks and valleys in the 1200-900 $cm^{-1}$ frequency range. The migration (dots) scale is zeroed at the beginning of the experiment and corresponds to an initial colony radius of approximately 3 mm. Spectra were collected with a Continuμm (Nicolet) microscope equipped with a MCT detector.

Fig. 2. To correlate EPS conformational changes observable between 2000 and 900 $cm^{-1}$ with aw variations observable from the 615 $cm^{-1}$ water librations band, spectra were collected with a NicPlan microscope equipped with a Si:B bolometer. A first PCA (A) was performed by using the whole spectral domain (2000-400 $cm^{-1}$) which again reflects major changes in the EPS absorption domain as shown in (B). A second PCA (C) was performed on the sole water absorption domain (900-400 $cm^{-1}$) which demonstrates that during active

swarming (here from 11h30 to 14h30), IR spectra exhibit positive PC1 loadings which correspond to low absorbencies at the low frequency and hence low aw. The data shown are from one representative experiment which has been reproduced on three distinct colonies.

Fig. 3. The figure displays 2D IR synchronous (A) and asynchronous (B) maps which clearly show that variations between the EPS and water domains are anti-correlated. The negative peak of the synchronous map (A) indicates that water decrease as EPS increases whereas the negative peak of the asynchronous map (B) indicates that the variations around 1000 $cm^{-1}$ (EPS) occur after the variations between 400 and 900 $cm^{-1}$ (water).

FIG 1

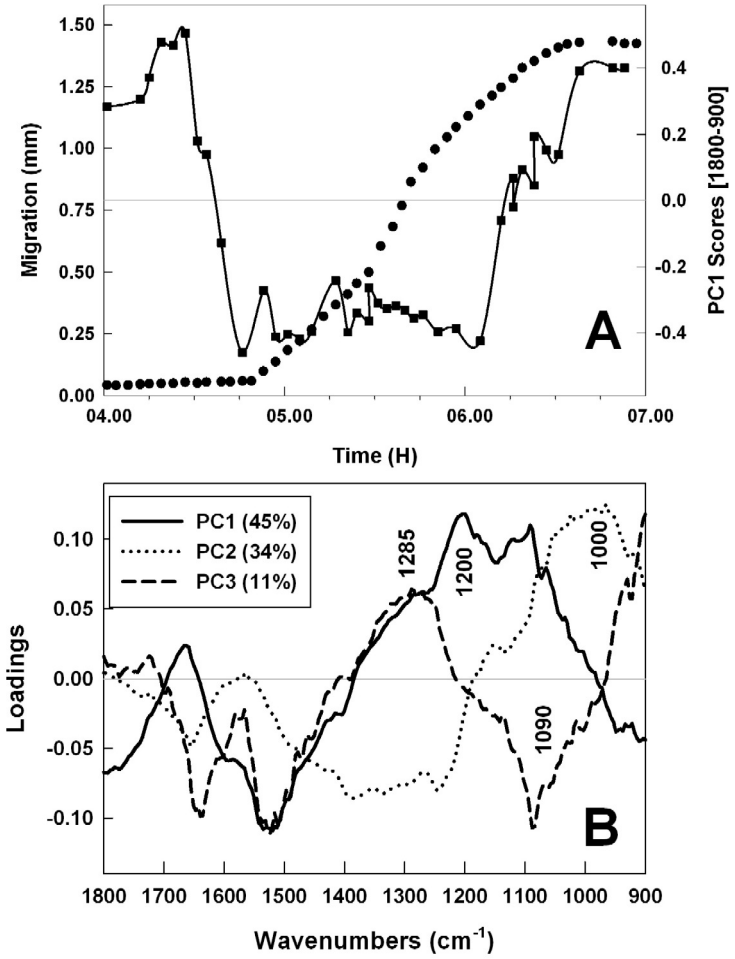


FIG2

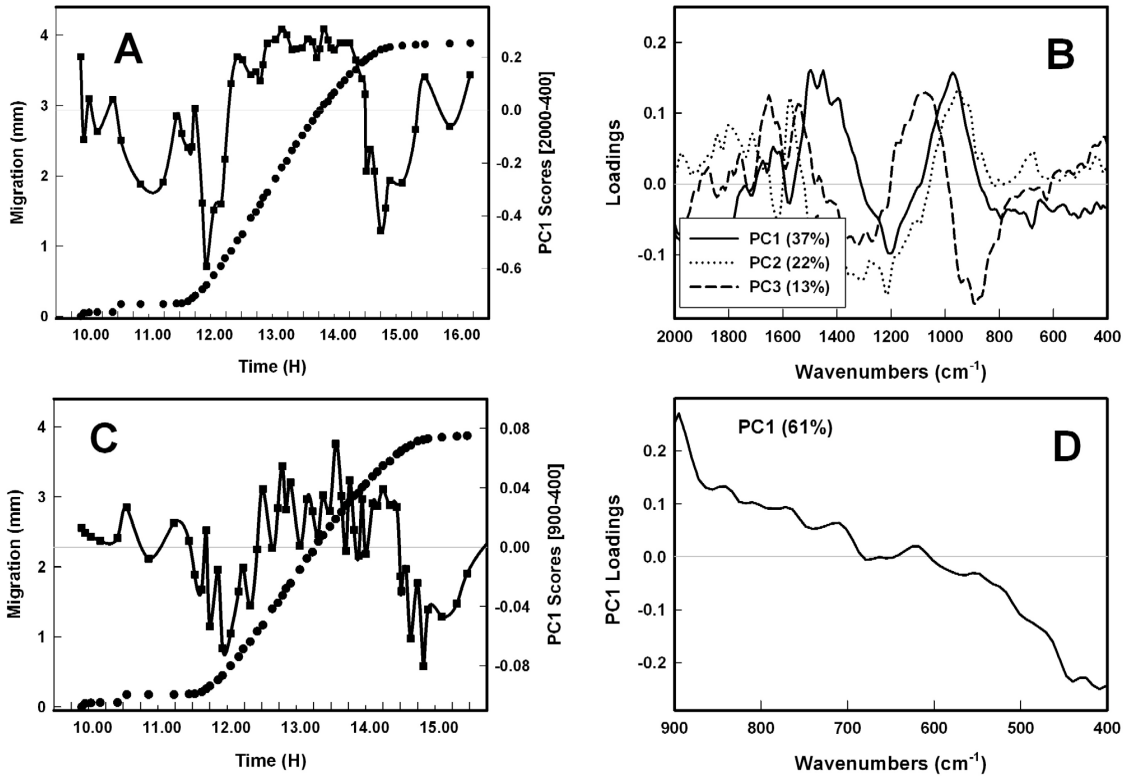


FIG3