Comparison of biosurfactant detection methods reveals hydrophobic surfactants and contact-regulated production

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Summary

Biosurfactants are diverse molecules with numerous biological functions and industrial applications. A variety of environments were examined for biosurfactant-producing bacteria including soil, water and leaf surfaces. Biosurfactant production was assessed with an atomized oil assay for a large number of bacterial isolates and compared with a commonly used drop collapse assay from broth and plate cultures. The atomized oil assay detected every strain that produced a biosurfactant detectable by the drop collapse test, and also identified additional strains that were not detected with the drop collapse assay because they produced low levels of surfactant or hydrophobic (low water solubility) surfactants such as pumilacidins. Not all strains that produced a biosurfactant detectable by the drop collapse test, and also identified additional strains that were not detected with the drop collapse assay because they produced low levels of surfactant or hydrophobic (low water solubility) surfactants such as pumilacidins. Not all strains that produced a biosurfactant detectable by the drop collapse test when cultured on agar surfaces produced surfactants detectable by drop collapse when cultured in broth, and vice versa. Many bacterial strains exhibited preferential production of surfactants when grown on an agar surface compared with broth cultures, and such surface enhancement of production could also be stimulated by increasing the viscosity of liquid culture media. Surface induction of surfactant pro-

Introduction

Biosurfactants, or biologically produced surface active agents, have received wide attention mostly for their potential for hydrocarbon dispersion and remediation. Bacterial biosurfactants were initially proposed to function as emulsifiers of biodegradable hydrocarbons (Neu, 1996). However, a wide variety of roles for biosurfactants have been since described, from biofilm formation to inhibitory activity against pathogenic organisms, sparking a renewed interest in their discovery (Ron and Rosenberg, 2001; Van Hamme et al., 2006). Given this interest in biosurfactants, the lack of knowledge of the distribution and frequency of occurrence of surfactant production in the environment is remarkable. Comprehensive examinations of biosurfactant production are lacking, and studies that have addressed this trait in a given environment can seldom be compared with those of other habitats (Perfumo et al., 2010); both the screening methods used, as well as pre-screening culturing conditions such as medium and incubation conditions usually vary widely between studies (Ahern et al., 2007; Hultberg et al., 2008).

In a recent report we described a high-throughput assay, which utilizes the application of atomized oil droplets to rapidly detect biosurfactants produced by bacteria on the surface of agar plates (Burch et al., 2010). This method has advantages over other common assays such as droplet collapse assays in that it can be performed for many colonies simultaneously after limited growth, does not require sample preparation of culture supernatants, and thus is suited for high-throughput screening for surfactant producing strains. Moreover, this method is capable of detecting much lower concentrations of surfactants than the drop collapse assay, and therefore in principle is capable of identifying biosurfactant producing strains that would escape detection with most other methods. However, since the atomized oil assay has not yet been tested on a broad range of environmental isolates, in this study we address whether the range of strains that it can detect includes all of those detectable
by the drop collapse assay. Furthermore, although the atomized oil assay has proven effective at detecting surfactants on agar plates, traditionally broth culture supernatants are screened for biosurfactant activity using the drop collapse assay. Depending on the properties of the surface-active compound and its biological role for the producing strain, its production may depend strongly on whether the producing cells are at a surface or not. Since a large difference in the transcriptomes of bacteria grown planktonically versus on surfaces have been described, with about one-third of genes differentially regulated (Schembri et al., 2003; Wang et al., 2004), it seems likely that biosurfactant production itself may be strongly influenced by cell culture conditions. Surface sensing is an important cue for many species to transition to surface-associated behaviour such as swarming, whereby cells move across a moist surface utilizing flagella and surfactant (Kearns, 2010). Although the surface regulation of flagella has been well documented (McCarter et al., 1988; McCarter, 2006), the regulation of surfactant production by surfaces has not yet been explored and will be addressed in this report.

A variety of isolated reports have described collections of biosurfactant producers from aqueous environments, polluted/unpolluted soils and even clouds (Bodour et al., 2003; Batista et al., 2006; Ahern et al., 2007; Maciel et al., 2007; Hultberg et al., 2008). Although the phyllosphere has not been the subject of such an investigation, it is widely assumed that plants are habitats that would be particularly selective for bacteria that produce surface active compounds (D’aes et al., 2010). In order to survive on leaf surfaces, epiphytes must be able to access limited and spatially heterogeneous nutrient supplies and endure daily fluctuations in moisture availability on a water-repellent surface (Hirano and Upper, 2000; Lindow and Brandl, 2003). Epiphytic bacteria could potentially use biosurfactants to increase the wettability of the leaf, to enhance diffusion of nutrients across the waxy cuticle, and/or aid in motility to favourable growth sites. Despite the substantial potential role of biosurfactants on leaves, only a few studies have examined their production in the phyllosphere, all of which have focused on their possible ecological role in only specific strains (Bunster et al., 1989; Hernandez-Anguiano et al., 2004; D’aes et al., 2010). A comprehensive examination of the phyllosphere inhabitants might reveal strains and biosurfactants not normally encountered in other habitats, and would address the hypothesis of phyllosphere enrichment of producing strains. In this study we compare the atomized oil assay with the drop collapse assay to characterize surfactants made by a broad collection of environmental strains, with an emphasis on epiphytes. We further demonstrate the usefulness of this assay in high-throughput screening and its much higher sensitivity for all types of biosurfactants encountered, many of which are hydrophobic and poorly detectable by the droplet collapse assay. We also investigate the influence of planktonic versus surface-associated culture conditions on the production of biosurfactants from our environmental isolates, and find evidence for frequent contact-dependent production of surface active compounds.

Results

Comparison of surfactant assays

A collection of 377 bacterial strains isolated from a variety of terrestrial and aquatic sources were grown on agar plates and tested for biosurfactant production using the atomized oil assay in which an airbrushed mist of oil droplets was applied to culture plates. Biosurfactant production was evident as a bright zone of de-wetted or raised oil droplets (hereafter referred to as a halo) (Fig. 1A). Additionally, cells of each strain suspended from plates into water as well as drops of broth culture supernatants were tested for drop collapse on an oil surface. A total of 33 of these strains exhibited biosurfactant production in at least one assay, yielding approximately 8.75% of all tested strains. Broken down by broad environment classes, 18 out of 150 (12%) of the total epiphytic isolates, 10 out of 124 (8%) of the total soil isolates, and 5 out of 103 (5%) of the total aquatic isolates demonstrated biosurfactant activity.

The identities of these strains were determined from partial 16S RNA sequences, and all isolates were
assigned to described taxa based on 98% BLAST sequence identity. Pseudomonas and Bacillus species were the most common genera identified, in line with previous reports of limited surveys (Bodour et al., 2003; Chen et al., 2007). All biosurfactant producers were members of the Gammaproteobacteria or Firmicutes except for a single Rhizobium species (Table 1). After eliminating duplicate taxa from the same sampling location, a total of 23 unique environmental strains that produced surfactant detectable in at least one assay were identified and further characterized (Tables 1 and S1). All 23 isolates produced surfactant detectable by the atomized oil assay, although only 16 isolates conferred drop collapse of either cells suspended from plates or of broth culture supernatants. Furthermore, cells of only nine of these 16 isolates conferred drop collapse from both culture conditions. Most of the other seven strains that conferred drop collapse only under one culture condition did so for suspended plate-grown cells. Pseudomonas syringae strains were typical of this group; cells of four representative isolates conferred drop collapse when suspended in water from plate cultures but not the supernatant of planktonic cultures. While 16 strains of P. syringae, P. fluorescens or B. subtilis produced biosurfactant that could be detected by both assays, the seven strains that exhibited biosurfactant activity that was detectable only by the atomized oil assay mostly consisted of a diversity of other taxa (Table 1).

**Hydrophobic biosurfactants**

Although not appreciated in most biological studies, surfactants differ greatly in their chemical properties in ways that could influence their ability to be detected by various assays. For instance, a fundamental property of a surfactant is its relative solubility in water and oil, which can be broadly described by its hydrophilic-lipophilic balance (HLB) value. Some important synthetic surfactants with low hydrophilicity are not readily dispersible in water, and thus have unique functions such as forming inverse emulsions of water into oil (Tadros, 2005). If a bacterial strain produced a biosurfactant with such low water solubility this could account for its inability to reduce the surface tension of water sufficiently to collapse a water drop. In order for drop collapse to occur on an oil surface, a minimum surface tension reduction at the water/air inter-

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- Strains that produced surfactants detectable with the atomized oil assay as well as conferring drop collapse in cells recovered from plates and in supernatants of broth cultures.
- Strains that produced surfactants detectable with the atomized oil assay, but which did not confer drop collapse irrespective of how cells were cultured.
- Strains that produced surfactants detectable with the atomized oil assay, but which only conferred drop collapse when cells grown on a plate were suspended in water.
- Strains that produced surfactants detectable with the atomized oil assay, but which only conferred drop collapse when supernatants of broth cultures were assayed.

face from 72 dyn cm\(^{-1}\) to around 43 dyn cm\(^{-1}\) is required (Bodour and Miller-Maier, 1998). Although a surfactant may be present in a sample of interest, it might not be detected by the drop collapse assay if it is produced in low quantities or has insufficient surface activity under the assay conditions to reach the threshold. Because the atomized oil assay can detect 10- to 100-fold lower concentrations of surfactant than that of the drop collapse assay (Burch et al., 2010), it is reasonable to hypothesize that the atomized oil assay can detect surfactant production in weakly producing strains. Therefore, it was possible that the seven strains that did not confer drop collapse may simply produce too little surfactant to be detected with this method. Indeed, many of these strains exhibited small halos in the atomized oil assay (Table S1), suggestive of low surfactant concentrations. However, a few strains such as Bacillus pumilis that did not cause drop collapse produced biosurfactants that conferred halos of de-wetted oil droplets around colonies that were at least as large as many strains whose biosurfactants did confer drop collapse (Fig. 1A). This observation led us to suspect that the surfactant had properties which hindered its ability to be detected by the drop collapse assay.

To address the features of biosurfactants that could be detected by the atomized oil assay but not the drop collapse assay, we distinguished the extent to which the atomized oil assay was responsible for their detection with the later method or whether the higher sensitivity of the surfactants might limit their detection. As a test of the relative hydrophobicity of the surfactants produced by Bacillus pumilis we suspended colonies of it and a few other strains such as Xanthomonas, Cedecaea and Rhizobium that exhibited the ability to de-wet atomized oil droplets but not to collapse water drops, retained the ability to de-wet oil droplets. This suggests that these strains produced only small amounts of a water-soluble surfactant that could be detected by the drop collapse assay if present in higher concentrations. In support of this conjecture was the observation that these later strains exhibited only relatively small halos in the atomized oil assay (Table S1). The low production of water-soluble surfactants in these strains was verified for P. syringae strain PB54 using mass spectroscopy (data not shown). This strain was observed to produce the same syringafactins as P. syringae B728a, albeit in much lower quantities, confirming that the detection of surfactants in strain PB54 by the drop collapse assay was compromised by its low level of production.

To confirm that the B. pumilis strain produced a surface active compound with low water solubility, we characterized the surfactant that it made by mass spectrometry. MALDI-TOF mass spectrometry was used to characterize the compounds from the cell-free region surrounding colonies on the surface of plates (Price et al., 2007; 2009). Agar plugs were vortexed with saturated 2,5-dihydrobenzoic acid (matrix) in acetonitrile, which was then directly used for mass spectrometry. The mass spectra of the plug extracts revealed a series of prominent peaks in the (m/z) range of 1050–1130 Da (Fig. 2), and were assigned as [M\(\text{Na}\)]\(^+\) and the [M+K]\(^+\) adduct ions of pumilacidin A, B, C and D (Naruse et al., 1990). The observed ions for the pumilacidins, the calculated molecular masses and the deduced molecular formulae are presented in Fig. 2. Several B. pumilis strains have previously been shown to produce a family of pumilacidins in this mass range (Naruse et al., 1990; Melo et al., 2009). We therefore conclude that our strain is producing...
a mixture of low water solubility pumilacidins that are capable of readily diffusing away from cells on the surface of an agar plate, but which are not sufficiently water soluble to impart drop collapse. To demonstrate the surfactant capabilities of the pumilacidins, the surface tension of a broth culture of *B. pumilis* was measured using a highly sensitive pendant drop analysis in order to detect weak activity. The surface tension of the broth culture supernatant was lowered by production of a surface active compound to 50 dyn cm\(^{-1}\); this surface tension is just above the minimum threshold necessary to impart a drop collapse.

Since the highly hydrophobic pumilacidins were detectable using the atomized oil assay, we further determined the efficiency with which other characterized synthetic surfactants differing in chemical properties could be detected by this method. The assay was performed on synthetic surfactants that possessed a broad range of hydrophobicities. As seen previously, the atomized oil assay readily detected surfactants having more balanced hydrophilic and lipophilic groups, which were also detected by the drop collapse assay (Table S2). On the other hand, the hydrophobic surfactants Span 85 and Span 80 each yielded large bright halos in the atomized oil assay, but given their low water solubility, could not be detected in the aqueous phase by the drop collapse assay (Table S2). This is in agreement with our observation that hydrophobic pumilacidins were also only detectable by the atomized oil assay and not by the drop collapse assay. Curiously, the synthetic surfactants not only caused bright halos of de-wetted atomized oil droplets, but those with balanced hydrophilic and lipophilic groups also caused the oil droplets to migrate away from the source of surfactant, travelling at a speed of up to 0.1 mm min\(^{-1}\) (Fig. S1). Such expanding halos may result from a strong surfactant gradient, such as explored by Angelini *et al.* (2009), although it is unclear why this should not be also conferred by the hydrophobic surfactants. This property was commonly observed around biosurfactant-producing bacterial colonies and might be used to infer the water solubility properties of the biosurfactants.

**Biosurfactants produced at a surface**

In addition to the surfactants that were only revealed by the atomized oil assay, we also found that many surfactants were detectable in the drop collapse assay only when cells had experienced a particular growth condition (Table 1). Most prominent among strains exhibiting such growth condition-dependent production of surfactants were strains of *P. syringae*; cultures of this species never conferred water drop collapse when grown planktonically. Two standard lab pathovars of *P. syringae*, DC3000 and B728a, were similarly tested and observed to also exhibit this pattern. Although *P. syringae* DC3000 has previously been reported to confer a drop collapse from broth culture, the authors did not employ a traditional drop collapse assay to determine this, but rather qualitatively observed the shapes of culture supernatant on parafilm (Berti *et al.*, 2007); when using the same strain of *P. syringae* DC3000, we observe that it does not produce sufficient quantities of syringafactin in broth culture to confer a true collapse on an oil surface. The factors determining surfactant production in *P. syringae* pv. *syringae* B728a, typical of this species, was thus investigated. While culture supernatants of this strain did not cause water drop collapse on an oil surface, plate-grown cells suspended to the same concentration as the planktonic culture conferred water drop collapse (Fig. 3A). Suspension of a syfA mutant blocked in production of syringafactin (Burch *et al.*, 2010) did not cause water drop collapse, confirming that the drop collapse is due to syringafactin. We thus postulated that enhanced expression of syringafactin production in cells grown on a surface was responsible. In order to link syringafactin production to surface-mediated increases in surfactant production, we


![Fig. 3. Contact-dependent production of syringafactin.](image)

A. Drop collapse assay of water alone (control), broth culture supernatant of *Pseudomonas syringae* B728a (broth), supernatant from an equivalent number of cells of *P. syringae* B728a that had been grown on an agar plate but then washed in water (plate), and supernatant from an equivalent number of cells of a syfA mutant of *P. syringae* that had been grown on an agar plate but then washed in water. B. Relative GFP fluorescence of cells of *P. syringae* B728a harbouring either a constitutively expressed GFP reporter gene (P519n–gfp) or a plasmid in which GFP expression is dependent on the promoter of SyfA (PsyA–gfp) recovered from broth and plate cultures.
examined the transcriptional regulation of syfA using a GFP-based bioreporter. Greater than a 10-fold increased expression of syfA was observed when cells were grown on an agar surface compared with planktonic growth in broth culture (Fig. 3B). As a control, a strain constitutively expressing GFP exhibited similar levels of fluorescence in both growth conditions. Thus, syringafactin is transcriptionally upregulated on agar plates; this probably explains the observed increased syringafactin yield from solid medium cultures of B728a (Burch et al., 2010), as well as the necessity of pooling 200 individual broth cultures in order to extract sufficient levels of syringafactin for metabolite characterization in DC3000 (Berti et al., 2007).

Since there have been reports that production of some surfactants are influenced by growth stage (Lin et al., 1994; Ochsner and Reiser, 1995), we examined syfA expression at a variety of times for up to 3 days during the growth of both liquid and solid cultures of *P. syringae*. GFP expression was higher in cells recovered from agar plates than broth cultures at all times, indicating that this is not growth-stage dependent phenomenon (data not shown). Additionally, some reports have documented that surfactant production is activated in more dense cultures by quorum sensing (Ochsner and Reiser, 1995; Lindum et al., 1998). However, the GFP fluorescence of *P. syringae* harbouring the *PsyfA–gfp* fusion in the wild-type and a quorum-sensing deficient strain (Quinones et al., 2005) was similar both in liquid and solid cultures, indicating that syringafactin production is not dependent on quorum sensing (data not shown). Although not previously connected to surfactant production, one of the ways by which bacteria sense surfaces is apparently through monitoring the viscosity of their environment (McCarter et al., 1988).

When PVP-360, a viscosifying agent, was added to broth medium, the expression of syfA was increased to levels similar to that of cells on agar plates (Fig. S2). Given this finding, we cultured the strains that had exhibited putative surface-dependent regulation of surfactant production for their ability to induce drop collapse when grown in viscous broth. While *P. syringae* B728a does not produce a surfactant capable of conferring drop collapse from normal broth cultures, it did so when grown in a viscous broth (Fig. 4B). A similar induction of surfactant production was induced by growth of other environmental strains of *P. syringae*, as well as *Pantoea* strain PB64 in viscous broth (Figs 4C and 4D). Interestingly, *P. fluorescens* strain PB59, which conferred a drop collapse only when cultured in broth medium, still produced abundant biosurfactant detectable by drop collapse when grown in viscous broth (data not shown), suggesting that its biosurfactant production is regulated by a different mechanism. Although it is tempting to speculate that the *P. syringae* and *Pantoea* strains are sensing a surface by directly measuring viscosity, growth in viscous broth could indirectly stimulate biosurfactant production via alteration of growth patterns such as cell aggregation, which was stimulated by the reduced turbulent drag of this culture medium. Vigorous shaking of *P. syringae* cultures reduced pellicle formation and resulted in a lower induction of syfA in viscous broth (data not shown).

**Discussion**

The application of the atomized oil assay to a wide variety of environmental bacterial strains and synthetic surfactants revealed it to be both more versatile and sensitive than the more commonly used drop collapse assay. The atomized oil assay confirmed surfactant production in every bacterial strain in which surfactants were detected using the drop collapse assay. More importantly, several bacterial strains were identified that produced either low amounts of surfactant or apparently hydrophobic surfactants that were not detectable using the drop collapse assay. The atomized oil assay readily confirmed biosurfactant production in taxa in which it had previously been described. The majority of the strains that produced surfactants detectable by both tests belonged to the genera *Pseudomonas* and *Bacillus* (14/16), both of which have been described in the literature to produce biosurfactants that lower the surface tension of water (Raaijmakers et al., 2010). The identification of such previously recognized surfactant-producing taxa emphasizes that while the drop collapse assay is suitable for finding such biosurfactant producers, the atomized oil assay may be more readily employed due to its high-throughput capability and higher sensitivity (Burch et al., 2010).

The atomized oil assay was particularly useful in identifying biosurfactant production in taxa in which this trait has not previously been shown or has been difficult to assess with traditional surfactant detection methods. For
example, our assay detected the hydrophobic pumilacidins produced by *Bacillus pumilis*, which have been documented for their potent antibiotic and antiviral properties (Naruse *et al.*, 1990), although their surfactant activity has previously been ignored due to their low water solubility (From *et al.*, 2007). Likewise, we detected surfactant production by a *Rhizobium* strain (Table 1); although we have not verified the compound, we suspect it could be similar to the long-chain AHLs produced by *Rhizobium etli*, which cannot be detected with a drop collapse assay but are documented as surfactants with a dual role in quorum sensing and swarming motility (Daniels *et al.*, 2006). Furthermore, a biosynthetic gene cluster proposed to synthesize a surface-active lipopeptide virulence factor was identified in the genome sequence of the plant pathogen *Xanthomonas axonopodis* (Etchegaray *et al.*, 2004); although incapable of imparting drop collapse, both an authentic culture of *X. axonopodis pv. glycines* as well as a related environmental strain found in this study produced compounds detectable with the atomized oil spray (Table 1, data not shown). Biosurfactants detectable only with the atomized oil assay were also observed in a *Ceddecea* strain, a taxon not previously known to produce surfactants; this feature may prove biologically important to its success as an opportunistic pathogen. Thus, the surface-active compounds that are produced by these strains that were detectable only with the atomized oil assay would have escaped attention in most other studies, but they may well have unique biological functions and/or potential industrial applications. Therefore, it appears that application of the atomized oil assay in environmental surveys might greatly expand our knowledge of novel biosurfactants.

While the atomized oil assay has many advantages over other assays there are some limitations that could bias the detection of surfactant producers. This assay best identifies bacterial strains that produce ‘bright’ halos around colonies (Fig. 1), although we have previously shown that some highly hydrophilic synthetic surfactants can modify oil droplets to appear ‘dark’ due to their flattened nature (Burch *et al.*, 2010). ‘Dark’ halos are less visibly obvious and no strains that unambiguously exhibited this appearance were found in our survey even though we approached the study with the expectation that we would find biosurfactants of this type. The spreading coefficient can be used to understand the behaviour of an oil droplet on a water surface (Fig. S3). The behaviour of an oil droplet is determined by the energy of the three interfaces present (water–air, water–oil, oil–air) and the surfactant’s effect on these interfacial energies. In the atomized-oil assay, the oil–air interfacial energy is constant, while the other two interfacial energies are altered by surfactants, if present. The surfactants absorb onto the interfaces in a manner that minimizes the overall energy of the system. If the surfactant is more effective in reducing the interfacial energy of the water–air interface relative to the water–oil interface, the area of the water–air interface will be maximized and the drop will rise and produce a bright halo. If the surfactant is more effective in reducing the interfacial energy of the water–oil interface relative to the water–air interface, the area of the water–oil interface will be maximized and the drop will spread out and produce a dark halo. A dark halo is most likely to be formed near a surfactant that has a large polar head group (or high HLB); this is because these surfactant molecules can pack more densely and energetically favourably to the curved water–oil interface versus the flat water–air interface. A dark halo thus likely indicates the presence of a surfactant with a large head group, and thus one that is probably relatively hydrophilic. We did not find any biosurfactants that yielded a water drop collapse and such a ‘dark’ halo. This is consistent with this class of surfactants being poor at reducing the water–air interfacial energy, which is important in the drop collapse assay. Another limitation of the atomized oil assay, which is shared with any culture-based assay, is that the nutrient medium that we used may have precluded us from detecting production of surfactants by some strains which require specific conditions for surfactant production. Furthermore, our assay is restricted to surfactant production by culturable organisms, although there is evidence that at least on leaves the most common cultured taxa are also among the most prevalent taxa identified by culture-independent methods (Yashiro *et al.*, 2011). Metagenomic investigation into the prevalence of biosurfactant production could be fruitful in expanding our understanding of their prevalence in bacterial communities, although advances will be limited until more genetic determinants for their production are described.

An unexpected finding from this study was that the production of surfactants that confered a reduction of surface tension was very conditional on whether the bacteria were grown on a surface or cultured planktonically. Although a number of studies have connected surface sensing with swarming motility (McCarter and Silverman, 1990; Harshey, 2003), we are only aware of one report, of *Serratia liquefaciens*, which has noted increased biosurfactant production in cells grown on a surface (Lindum *et al.*, 1998). In the current work we have shown that a surprisingly large proportion of bacterial strains restrict biosurfactant production to growth on a surface. Although most of these surface-dependent surfactant producers were strains of *P. syringae* isolates, this phenomenon was also seen in a *Pantoaea* strain, suggesting that it may be a common trait. Commonly used methods of screening for biosurfactants by drop collapse employ broth cultures and would likely not identify such strains. On the other hand, two strains were identified that only conferred drop col-
lapse from broth culture and not from cells grown on plates and subsequently suspended in water drops. However, surfactant production was still detectable in these strains as a small halo of de-wetted oil droplets with the atomized oil spray when cells were grown on plates. The small halo size of these two strains indicates that the amount of surfactant produced by cells grown on plates was probably too low in concentration to be detected by the drop collapse assay; therefore surfactant production was not fully blocked at a surface, but rather dramatically reduced. Although we have not yet encountered such strains, there is the potential for us to overlook biosurfactants which are produced exclusively in broth culture. However, such strains must be uncommon based on our extensive survey, and the high sensitivity of the atomized oil assay should enable even very low production on solid surfaces to be detectable.

Presumably, the strong environment-dependent regulation of surfactant production at surfaces is linked to its role in the habitat of some strains. For example, surfactants contributing to biofilm growth or movement on a surface would be pointless if produced in an aqueous environment. Thus, it makes sense that bacteria with multiple habitats should survey their growth environment before committing to production of a biosurfactant. The surface trigger for surfactant production and its conservation among bacterial taxa remains an active area of research. Bacterial surface sensing has been coined the “holy grail” of swarming motility research” (Kearns, 2010). A few specific mechanisms for surface sensing have been investigated, such as two-component systems and flagellar inhibition (Otto and Silhavy, 2002; Belas and Suvanasuthi, 2005). It is intriguing that increases in viscosity led to increases in surfactant production in this study (Fig. 4), much as it has been shown to induce production of flagella in Vibrio parahaemolyticus (McCarter et al., 1988). However, our results lead us to believe that it is not viscosity sensing per se that is inducing surfactant production, but rather perception of a growth pattern such as cell aggregation that perhaps restricts movement of cells which, in turn is induced by the reduced turbulent drag of a viscous medium. Although it is tempting to speculate that oxygen sensing is involved, neither the high oxygen condition of a shaken culture, nor an oxygen starved broth culture is sufficient to induce syringafactin production (data not shown). We are currently investigating the mechanism behind surface regulation of syringafactin in P. syringae B728a, and the biological purpose for restricting syringafactin production to surfaces.

In addition to suggesting that surfactant producers often restrict production to surfaces, our broad collection of isolates also reveals the phyllosphere to be a rich source of biosurfactant producers. If a detailed analysis confirms that certain environments such as waxy leaf surfaces are particularly enriched in biosurfactant producers, this might further elucidate the natural functions of biosurfactants. We are actively utilizing the atomized oil assay to determine the distribution of culturable biosurfactant producers in the phyllosphere and other environments.

**Experimental procedures**

**Bacterial strains and growth conditions**

Bacteria were isolated on 10% Trypticase Soy Agar (Difco, Detroit, MI, USA) containing 1.5% agar and natamycin. Both P. syringae pv. syringae B728a (Loper and Lindow, 1987) and environmental strains were maintained and screened for surfactant production on King’s medium B (KB) (King et al., 1954) and grown at 28°C. Viscous KB broth was produced by amending with polyvinylpyrrolidone (PVP-360) to a concentration of 10% W/V (McCarter et al., 1988). Antibiotics were used at the following concentrations (µg ml⁻¹): natamycin (21.6), kanamycin (50) and spectinomycin (100).

**Environmental isolates**

A total of 377 isolates were obtained over the course of one year from diverse locations in California. Half the isolates were from plant samples while the remainder were from soil and water samples collected in native California Chaparral habitats. Serial dilutions of the samples were incubated for 4–5 days, and only morphologically distinct taxa from each sample were chosen for testing.

**Biosurfactant detection assays**

The drop collapse assay was performed as according to Bodour and Miller-Maier (1998). Two microtubes of 10W-40 Pennzoil (Pennzoil Products Company, Houston, TX, USA) was applied to delimited wells on the lid of a 96-well plate and allowed to equilibrate at room temperature. Next, 5 µl of either diluted surfactant samples or supernatant from bacterial cultures or re-suspended bacterial colonies was pipetted onto the oil surface. Bacterial broth cultures were grown for two or more days until the cultures reached an OD₆₀₀ of at least 1, while bacterial plate cultures were grown for two days and resuspended in phosphate buffer (10 mM, pH 7.5) to an approximate OD₆₀₀ of 1. Drops which retained a spherical shape were scored as negative for surfactant content, while drops which had a visibly decreased contact angle with the oil and spread (collapsed) were scored as positive for surfactant content.

The atomized oil assay was conducted as follows: Bacteria were evenly spotted onto KB agar plates using sterile toothpicks and grown overnight; longer incubation periods result in overlapping surfactant halos which can mask surfactant production by neighbouring strains. When necessary, individual strains with slow growth rates or potentially masked surfactant halos were re-spotted onto separate plates and allowed extra time for growth and surfactant production before testing with the atomized oil assay. Alternatively, if visualizing surfactant from broth culture, 1 ml of 2-day-old broth culture was...
centrifuged at 10 000 g for 2 min, and 5 μl of supernatant was pipetted onto the plate and allowed to equilibrate for 30 min before assaying. Halo sizes are dependent on both surfactant quantity and diffusion time (Burch et al., 2010), and thus surfactant quantity cannot be directly compared between these two conditions. Synthetic surfactants were similarly pipetted onto plates. An airbrush (Type H, Paasche Airbrush, Chicago, IL, USA) was used to apply a fine mist of mineral oil (light paraffin oil, Fisher Scientific) onto the plate with an air pressure between 15 and 20 psi. Biosurfactant halos were then immediately visualized with an oblique source of bright light.

Matrix-assisted laser desorption ionization mass spectroscopy

The mass spectra were acquired on a Bruker Daltonics Omni flex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) operating in reflectron mode. Cellular colonies and agar plugs were vortexed with saturated 2.5-dihydrobenzoic acid (matrix) in acetonitrile, and aliquotted onto a standard 49-place target at room temperature prior to introduction into the instrument. Ion source 1 was set to 19.0 kV, and source 2 to 14.0 kV, with lens and reflector voltages of 9.20 and 20.00 kV respectively. A 200 ns pulsed ion extraction was used with matrix suppression up to 250 Da. Excitation was at 337.1 nm, typically at 60% of 150 μJ maximum output, and 80 shots were accumulated. The reflectron mass resolution (FWHM) for m/z 2465 (ACTH 18–39) was > 20 000. The instrument was calibrated externally on a dp series of malto-oligosaccharides.

Surface tension measurements

The surface tension of cell-free supernatants was determined using the pendant drop method. Cell-free supernatants were analysed with a FTA 4000 video analysis instrument (First Ten Angstroms, Portsmouth, VA, USA). Droplets were produced using a 22-gauge blunt needle and the values reported represent an equilibrium surface tension determined 60 s after drop formation.

Measurement of gene expression

Wild-type P. syringae B728a carrying either a plasmid conferring constitutive fluorescence (p519ngfp, Matthesyse et al., 1996) or a plasmid indicative of transcription of the syringafactin biosynthetic locus (pPsyrA–gfp, Burch et al., 2010) was grown in KB media overnight, then suspended in phosphate buffer (10 mM, pH 7.5) to an approximate OD600 of 0.2. GFP fluorescence intensity was determined using a TD-700 fluorometer (Turner Designs, CA, USA) with a 486 nm bandpass excitation filter and a 510 to 700 nm combination emission filter. A relative fluorescence unit was defined as the fluorescence of the suspensions normalized for the suspension turbidity measured as OD600.

Bacterial identification

Genes encoding 16S rRNA were amplified by colony PCR using universal primers 27F (5’-AGAGTTGTATCCTGCGC-TCAG) and 1492R (5’-TACGCTACCTTGTATCGAGCTT) (Lane, 1991). PCR conditions were as follows: an initial denaturation of 10 min at 95°C, 28 cycles of 95°C, 59°C, and 72°C for 1 min each, with a final extension time of 10 min at 72°C. Products were excised from an agarose gel, extracted (UltraClean GelSpin, MoBio, CA, USA), and submitted for sequencing at the UC Berkeley Sequencing Facility. The 16S rRNA gene sequences were compared with GenBank databases using BLAST (http://www.ncbi.nlm.nih.gov). Sequences having > 98% similarity to a known GenBank sequence were assigned to the designated phylotype. These sequence data have been submitted to the GenBank database under accession numbers JF430870–JF430892.

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References


Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Time-elapsed photomicrographs of oil droplets constituting an ‘expanding’ halo near a droplet of Triton N-57 placed on an agar plate when examined at 0 (A), 1 (B), 2 (C), 3 (D), 4 (E) and 5 (F) minutes after atomizing oil droplets onto the surface of the agar plate. The white spot is a fixed reference point that allows visual orientation of the starting location of one of the moving oil droplets. The bar represents 0.1 mm.

**Fig. S2.** Relative fluorescence of cells of *P. syringae* B728a harbouring either a plasmid conferring constitutive fluorescence (P519n–gfp) or one in which GFP expression is dependent on the promoter of SyfA (PsyfA–gfp) that were recovered from KB broth or viscous KB broth cultures in which PVP-360 (10% W/V) had been added.

**Fig. S3.** Forces acting on an oil droplet on a water surface and how the shape of the droplet can be determined from the spreading coefficient equation, where $S =$ the spreading coefficient, $\gamma$ is the interfacial energy of the water (w), air (a) and oil (o) interfaces.

**Table S1.** Characteristics of biosurfactant-producing bacterial strains.

**Table S2.** Comparison of the behaviour of a variety of synthetic surfactants in the atomized oil and drop collapse assays for surfactants.

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