Identification and characterization of a highly motile and antibiotic refractory subpopulation involved in the expansion of swarming colonies of *Paenibacillus vortex*

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Summary

Bacteria often use sophisticated cooperative behaviours, such as the development of complex colonies, elaborate biofilms and advanced dispersal strategies, to cope with the harsh and variable conditions of natural habitats, including the presence of antibiotics. *Paenibacillus vortex* uses swarming motility and cell-to-cell communication to form complex, structured colonies. The modular organization of *P. vortex* colony has been found to facilitate its dispersal on agar surfaces. The current study reveals that the complex structure of the colony is generated by the coexistence and transition between two morphotypes – ‘builders’ and ‘explorers’ – with distinct functions in colony formation. Here, we focused on the explorers, which are highly motile and spearhead colonial expansion. Explorers are characterized by high expression levels of flagellar genes, such as *flagellin* (hag), *motA*, *fliI*, *flgK* and *sigD*, hyperflagellation, decrease in ATP (adenosine-5′-triphosphate) levels, and increased resistance to antibiotics. Their tolerance to many antibiotics gives them the advantage of translocation through antibiotics-containing areas. This work gives new insights on the importance of cell differentiation and task distribution in colony morphogenesis and adaptation to antibiotics.

Introduction

Bacterial swarming is a rapid and coordinated migration of bacteria across surfaces (Harshey, 2003; Kearns, 2010). Powered by flagella and often facilitated by surfactant secretions, the swarm generally contains correlated moving cells that stream in multiple directions, forming the classical long-lived patterns of whirls and jets (Darnton et al., 2010; Zhang et al., 2010). The soil bacteria *Paenibacillus vortex* is an example of a peritrichously flagellated species that exhibits a different type of swarming pattern while spreading on hard agar surfaces (1.5–2.25% w/v). *P. vortex* generates leading groups of cells named vortices that cooperatively whirl for long times (order of hours) around a common centre. The vortices expand in size and move outward from the inoculation point, leaving behind a trail of cells, resulting in a tendril-like colonial structure (Cohen et al., 1996; Ben-Jacob, 1997; 2003; Ben-Jacob and Cohen, 1997; Ben-Jacob et al., 1997; 1998). The vortices serve as building blocks for new colonies.

*P. vortex* is capable of collectively crossing regions containing a large variety of antibiotics by using ‘pioneering, swarming masses’ (Ingham et al., 2011). After reaching an antibiotic-free area, the pioneering bacteria regain their sensitivity to the same antibiotic (Ben-Jacob et al., 2000; Ben-Jacob, 2003; Ben-Jacob et al., 2004), suggesting temporary phenotypic adaptation. Adaptability to antibiotics and to changing environments can be achieved in colonies with task distribution and cell differentiation. Similar phenomena are found in persister cells in *Escherichia coli* (Balaban et al., 2004; Lewis, 2010).
phenotypic switching between motile rods and robust cocci in *Paenibacillus dendritiformis* (Be’er et al., 2011), and the formation of biofilms in *Bacillus subtilis* (Stewart and Costerton, 2001). However, for *P. vortex*, the mechanism is unclear. Here, we show that *P. vortex* colonies are composed of two coexisting morphotypes [bacterial phenotypes, display characteristics of distinct colonial morphologies (Ben-Jacob et al., 1998)], referred as ‘builders’ and ‘explorers’. Under normal growth conditions, the builders constitute the majority of the population; they are recognized by a relatively high growth rate in liquid and reduced swarming capabilities on solid medium. The explorers, on the other hand, have a reduced growth rate in liquid and are hyperflagellated, with elevated swarming capabilities on hard surfaces. The population is, thus, maintained by fast reproduction and expansion rates. Explorers are in the minority during liquid growth and are mainly found in the leading vortices on solid surface; however, they are extremely resistant to antibiotics, thus maintaining the group under antibiotic stress. The coexistence of these two subpopulations provides a unique advantage for colony growth. Our findings may have a general relevance to bacteria living in challenging and changing environmental niches, such as soil and rhizosphere.

**Results**

***Isolation of explorers and builders***

A colony of *P. vortex*, spreading from the inoculation point on a hard agar, has the following structures: vortices at the leading edges, branches behind them and an inoculation zone at the centre (Fig. 1A). It has been shown that colonies initiated from bacteria taken from different structures of the colony generate distinct patterns (Ben-Jacob et al., 2004; Ben-Jacob and Levine, 2006). Following these observations, we isolated cells from two different structures: the vortices and the centre. The resulting colonies had distinct colonial morphologies with enhanced (1.5-fold) and no expansion rates, respectively (Fig. 1B and C), compared with normal expansion rate on peptone plates (Fig. S1A and B).

To test whether distinct colonial morphologies resulted from different functional properties of different morphotypes, we sorted and separated the population based on hypothetical tasks distribution of the bacteria in the colony.

***Isolation of explorers.*** Since vortices are located at the leading edge of the colony, we assumed that a vortex’s population might be more tolerant to different stresses, such as antibiotics. To test this hypothesis, *P. vortex* cells were treated with kanamycin (10, 20 and 40 μg ml⁻¹) for 18 h. Pre-exposed cells were spread over antibiotic-free Luria–Bertani (LB) agar, and microcolonies were picked to measure the expansion diameter on agar, and the growth rate and the yield in liquid (20 h) (Fig. 2A). With the increase in kanamycin concentration (10, 20 and 40 μg ml⁻¹), there was a decrease in both the growth rate (1.2-, 2.2-, 6.4-fold, respectively) and the growth yield in LB liquid (1.6-, 1.7-, 2-fold, respectively), in comparison to a mixed culture (untreated culture, composed of all morphotypes) (Fig. 2C). However, the expansion diameter of the resulting colonies surprisingly increased with the additions of kanamycin (more than 1.4-fold) (Figs 2B and S1). Since this population tended to spread fast and seemed to be found mainly at the leading edge of the swarming colony, we referred this morphotype as explorers.
Isolation of explorers. Searching for additional sub-populations that may comprise *P. vortex* colonies, we exploited the explorers’ slow growth rate (Fig. 2C) and used repeated dilutions growth cycles to select against this phenotype (as described in Keren *et al.*, 2004 for the elimination of persisters). The culture was grown in LB liquid O.N, diluted 1:100 into fresh LB and grown for 4 h, then diluted again as above for three more times (Fig. 2D). After each dilution, the culture resistance was tested immediately with 20 μg ml⁻¹ kanamycin, followed by a viable count on LB agar. After the fourth dilution, no growth was detected following exposure to kanamycin, indicating that kanamycin-resistant bacteria were no longer present in the culture, or at least extremely rare (data not shown). After four dilutions, the bacteria were spread over LB agar, and microcolonies were picked (immediately after their appearance – 10 h) to measure the expansion diameter, as well as the growth rate and yield in liquid (20 h). The resulting lineage developed colonies with reduced expansion diameter (1.5-fold) on LB plates (Fig. 2E I and II), and elevated growth yield (1.5-fold) and growth rate (1.75-fold) in liquid LB (Fig. 2F). Testing the response to antibiotics, we found that the cells enriched by four dilution cycles were less (in comparison with the explorers) resistant to kanamycin both in liquid [minimal inhibitory concentration (MIC) of explorers < 7.8 μg ml⁻¹, *builders* < 62.5 μg ml⁻¹] and solid LB (Fig. S2). This morphotype was called *builders* as they seemed to be most common in the interior and appear to form the most common morphotype in the mature colony.

Since changes in growth rate and growth yield (20 h) in liquid medium and expansion diameter on solid surface were the most distinguishable characteristics in both explorers and builders, for further characterization and detection of the morphotypes, we used these parameters (Fig. S3, Table S1). Cells that have presented an increase of ≥ 10% in expansion diameter and a decrease of ≥ 10% in the absorbance after 20 h in comparison to mixed culture were defined as explorers.

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microcolonies were picked for evaluation of explorers’ percentage. At $t = 0$, explorers comprised one third of the culture (Fig. 3B). As the population proliferated in the presence of kanamycin, the fraction of explorers appeared to increase and appeared to constitute >99% of the culture after 18 h. During the first 4 h of culture in the presence of kanamycin, the total number of viable bacteria decreased (presumably, being killed or inhibited by the antibiotic), while the percentage of explorers increased, from 31% at $t = 0$ reaching 56% (10 $\mu$g ml$^{-1}$), 75% (20 $\mu$g ml$^{-1}$) and 81% (40 $\mu$g ml$^{-1}$) at $t = 4$ h (Fig. 3A and B). Thus, the explorers are constantly present as a significant minority of P. vortex’s population, being enriched in the presence of kanamycin.

The morphotypes stability

We have noticed that builders’ culture, obtained formerly by repeated dilutions at the early log phase, was unstable and changed within a few hours into a mixed culture. To test builders’ stability, we evaluated the time it takes for a population of builders to return into a mixed culture. To do so, purified builders were allowed to grow in liquid medium for 24 h and were sampled at different time points. The percentages of builders and explorers were evaluated using identification parameters presented at the first section. Builders were the only morphotype detected in the culture 8 h post-inoculation (Fig. 4A II). However, after 12 h, builders composed only of 50% of the culture (similar to mixed culture) (Fig. 4A III). After 24 h of incubation, the percentage of builders remained the same (Fig. 4A IV). Builders plated on LB agar reverted back to mixed colony within 24 h (data not shown). Cells isolated from each time point and exposed to kanamycin showed no growth after 8 h, and showed similar growth rate in comparison to mixed culture after more than 12 h (Fig. S4). Thus, builders’ culture remains stable for at least 8 h until explorers reappear within ≥12 h in liquid and solid media respectively.

One explanation of explorers’ appearance in the purified builders’ culture may be due to a rapid division of a few remaining explorers. To calculate the mean generation time of explorers that might have been left in the culture after dilution, we used the following equations:

$$n = \frac{\log N_t - \log N_0}{\log 2}, \quad G_t = \frac{t}{n}$$

The number of divisions ($n$) was determined by dividing the log of the number of cells ($N_t$) at time $t$ minus the log of the number of cells at time zero ($N_0$), by log2. The mean generation time ($G_t$) was calculated by dividing time ($t$) by the number of divisions ($n$).

Under normal growth conditions, the mean generation time ($G_t$) of a P. vortex culture (over 12 h) is approximately
2 h. However, according to estimation, the $G_t$ needed for a hypothetical fraction of 0.1% ($1 \times 10^3$ CFU/ml) explorers to reach 50% of the culture ($1.5 \times 10^8$ CFU/ml) in 12 h is 42 min; 1% explorers will reach the same concentration in 52 min. Thus, the estimated $G_t$ is much shorter than the observed $G_t$. This indicates that the explorers probably emerged by a phenotypic transition rather than by replication.

The stability of explorers was tested by several approaches: First, isolated explorers were grown to high density (48 h) in liquid medium without kanamycin. The percentage of explorers in the resulting culture was 95% (Fig. 4B I). Second, isolated explorers were grown in liquid culture for 24 h, re-inoculated for another 24 h growth. After five such transfers, the resulting culture contained 90% explorers (Fig. 4B II). Finally, isolated explorers were inoculated on a fresh LB agar. The resulting colony (24 h growth) contained 60% explorers only (Fig. 4B III). The explorers morphotype was shown to be extremely stable in liquid, meaning almost no reversion to the mixed culture occurred. However, when explorers were inoculated on solid surface and formed a colony, only 60% explorers remained, that is the transition to mixed culture has occurred showing typical growth rate and swarming capabilities (not shown). We have shown that both morphotypes can develop into the other; their characteristics are stable. However, the stability of these morphotypes is different. We further focused on explorers because of their stability and importance of phenotypic resistance to antibiotics.

Explorers are hyperflagellated

To test if the enhanced swarming of the explorers is supported by hyperflagellation, we examined the level of flagella synthesis. First, we tested the expression of flagellin and motility-related genes by real-time PCR (polymerase chain reaction) using primers amplifying the following genes: flagellin (hag), flgK, fliI, motA and sigD (hypothesized annotations of flagella genes presented in Table S4). These genes were selected based on the outcome of the Genome Holography bioinformatics analysis of microarray chip results (Madi et al., 2008; Roth et al., 2011). For more details, see Figs S5 and S6, and Table S2. The analysis was done to provide a functional representation of flagella biosynthesis genes. Comparing the expression values between mixed culture and isolated explorers, we found that the explorers exhibit more than a 1.5-fold elevation in the expression of flagella and motility-related genes (Fig. 5A). We also found that the expression levels increased with kanamycin concentration (pre-exposure to 10–40 μg ml$^{-1}$ kanamycin) (Fig. 5A).
Second, flagellin levels were quantified in explorers and mixed cultures by Western blot. Two types of antibiotics were used for these experiments to verify if the phenomenon was unique to kanamycin or more general. Quantitative Western blot was performed on extraction of flagella proteins from equal CFU of explorers, isolated from either 40 μg ml⁻¹ kanamycin or 15.6 μg ml⁻¹ chloramphenicol for 18 h, and mixed cultures using anti-flagellin antibody. ImageJ software (National Institutes of Health, Bethesda, MD, USA) and total protein analyses have shown elevation of approximately 2.2-fold and more than 1.2-fold in flagellin extracted from explorers isolated from chloramphenicol and kanamycin, respectively, in comparison to mixed culture (Fig. 5B).

C. TEM imaging. Increase of flagella filaments in explorers in comparison to builders. Scale bar = 2 μm.

Fig. 5. Explorers’ are hyperflagellated.
A. Real-time PCR. Increase of flagella gene expression is in correlation to kanamycin concentrations. Explorers were isolated after pre-exposure to 10 (white), 20 (light grey) and 40 μg ml⁻¹ (dark grey) kanamycin for 18 h, and examined relatively to the mixed culture. Y-axis represents the binary logarithm of genes relative expression (ΔΔCt). X-axis: sigD, fliI, flgK, motA and flagellin (hag).
B. Western blot with anti-flagellin antibody. Elevation of flagellin in explorers isolated from either 40 μg ml⁻¹ kanamycin (Kan t) or 15.6 μg ml⁻¹ chloramphenicol (Cl t). Non-treated bacteria (Cl c, Kan c). The number of cells in all samples (10¹⁰ CFU/ml) was equalized; thus, the levels of proteins presented here are per cell.
C. In the presence of Congo red, flagella production is inhibited. Mixed culture and explorers were plated on agar plates with 20 μg ml⁻¹ kanamycin (I and II) and 20 μg ml⁻¹ kanamycin + 400 μg ml⁻¹ Congo red (III and IV) and incubated for 24 h. Absence of flagella in the presence of Congo red prevented swarming of the mixed as well as explorers colonies. Scale bar = 5 mm.

Phenotypic flagella testing by transmission electron microscope (TEM) imaging using explorers samples isolated from the same antibiotics as in previous paragraph has shown similar results. Explorers were found to have significantly more flagella compared with mixed culture (Fig. S7). Finally, TEM images of explorers and builders taken from microcolonies grown for few hours on LB plates have shown elevated number of flagella in explorers in comparison to builders (Fig. 5C). These results confirm that explorers are hyperflagellated.

However, it has been previously suggested that P. vortex may have additional ways to move on solid surface (i.e. with the aid of pili) (Sirota-Madi et al., 2010). To test whether flagella are essential for colony expansion on solid medium, we have grown explorers on plates with 400 μg ml⁻¹ Congo red (known to inhibit flagella production) (Arnold and Shimkets, 1988; Velicer et al., 1998) with and without 20 μg ml⁻¹ kanamycin. Congo red has previously been shown to inhibit flagellar motility of P. vortex at this concentration (Ingham and Ben-Jacob, 2008). In a
Resistance to kanamycin is negatively correlated with ATP levels. Explorers were isolated from 10, 20, 40 μg ml⁻¹ kanamycin (x-axis).

A. The resistance of explorers to kanamycin was examined using disk diffusion assay containing 50 μg kanamycin. The bars represent the averaged radius of inhibition zone, which indicates the resistance level of explorers (P < 1 × 10⁻⁴) (i.e. smaller radius indicates higher resistance). The resistance increases with the increase in kanamycin concentrations during the pre-exposure.

B. The bars show the level of cellular ATP of explorers isolated from similar concentrations of kanamycin (P < 0.05). The assay was performed using ATPlite kit. There is a negative correlation between ATP cellular levels and kanamycin concentrations.

Fig. 6. Resistance to kanamycin is negatively correlated with ATP levels. Explorers were isolated from 10, 20, 40 μg ml⁻¹ kanamycin (x-axis).

Since the concentrations of kanamycin during the pre-exposure are positively correlated with explorers' resistance (Fig. 6A) and negatively correlated with cellular ATP levels. These findings are in agreement with the resistance to kanamycin observed in bacteria with reduced cellular ATP (e.g. small colony variants, persisters), and with negative correlation found between growth rate and kanamycin concentration (Fig. 2C).

The tolerance of explorers to other classes of antibiotics

To test if the explorers' resistance is restricted to kanamycin or is a general reaction to antibiotics, we further examined the tolerance of explorers to six other types of antibiotics that represent five distinct antibiotic families with different modes of action (kanamycin, spectinomycin, chloramphenicol, novobiocin, rifampicin and ampicillin; Table S5). P. vortex bacteria were exposed to the MIC of each antibiotic for 18 h. The expansion diameter on LB agar, growth rates and growth yield (20 h) of pre-exposed bacteria were examined. The results of pre-exposure to different types of antibiotics were similar to pre-exposure to kanamycin (isolation of explorers): wider expansion diameter on solid medium (Fig. 7A–G), and decreased growth rates and growth yields after 20 h of bacteria in liquid medium (Fig. 7I–VI). These findings may indicate that explorers' responses to antibiotic stress are not unique to kanamycin but also applies to a wide range of structurally unrelated antibiotics.

In another set of experiments, we tested the tolerance of explorers to different types of antibiotics incorporated into agar plates (isolation of explorers). Explorers were isolated from different concentrations of kanamycin (10, 20, 40 μg ml⁻¹), were plated on LB agar. The resistance of explorers to kanamycin was indicated by inhibition zone caused by a disk containing 50 μg kanamycin placed at the centre of the plate (Fig. 6A). We found that explorers isolated from higher concentrations of kanamycin have shown an increased resistance (Fig. 6A).

It has been previously shown that resistance to aminoglycosides (kanamycin), accompanied with reduced growth rate in liquid, is often associated with a reduction in membrane potential (proton motive force, PMF) and ATP levels due to defects in the electron transport chain (Wilson and Sanders, 1976; Proctor et al., 1998; Melter and Radojevic, 2010). Motivated by this connection, we tested ATP levels in the mixed and explorers cultures to see if there is dependence between cellular ATP and resistance to kanamycin. We found a decrease of approximately 10%, 30% and 45% in cellular ATP levels for the explorers isolated from 10, 20 and 40 μg ml⁻¹, respectively, in comparison to the mixed culture (Fig. 6B). Since the concentrations of kanamycin during the pre-exposure are positively correlated with explorers' resistance (Fig. 6A) and negatively correlated with cellular ATP levels (Fig. 6B), one may conclude that the resistance to kanamycin is negatively correlated with ATP levels. These findings are in agreement with the resistance to kanamycin observed in bacteria with reduced cellular ATP (e.g. small colony variants, persisters), and with negative correlation found between growth rate and kanamycin concentration (Fig. 2C).

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in agar. Explorers isolated from different types of antibiotics were inoculated on LB plates and examined using disk diffusion assays. The disks used in each assay contained the same antibiotics as the former pre-exposure. In all cases, explorers were tolerant to the same antibiotics during secondary exposure, having a smaller radius of inhibition than the control (Fig. 7H–S).

Discussion

This study reveals a novel aspect in the morphogenesis of bacterial colonies and adaptation to antibiotics. Since P. vortex cannot yet be studied using genetic means (it is not currently transformable), we have used other methods to distinguish and investigate P. vortex’s subpopulations. We have shown that P. vortex cultures can contain at least two coexisting subpopulations (‘builders’ and ‘explorers’) with different task distribution that together form a unique colony pattern. The expansion patterns of cells isolated from different locations of P. vortex colony (centre and vortex) (Fig. 1) implies that builders comprise mainly the internal parts of the colony, while explorers are located mostly in the periphery of the colony. The existence of explorers and builders at distinct locations of the expending colony is consistent with the model explaining swarming migration involving elongation of dendrites in B. subtilis. When swarvers accumulate at the tip of the dendrite establishing a leader track and non-swarvers that group at the edge of the inoculation zone build the dendrite by growth and multiplication (Matsuyama and Matsuyama, 1993; Hamze et al., 2011).

The phenomenon of two distinguishable cell types that coexist within the clonal population is sometimes referred to as ‘bistability’. Bistability is exemplified in E. coli by persister cells, and in B. subtilis by genetic competence, spore formation/cannibalism and swimming/chaining. Bistability might be advantageous, allowing cells to hedge their bets so that a few cells enter a state that would be better adapted to one circumstance or another should that circumstance arise (Dubnau and Losick, 2006). The coexistence of explorer and builder morphotypes as subpopulations suggests that their transition is also bistable, although we do not exclude further morphotypes. The proportion of the two morphotypes in the culture can be changed, and both morphotypes can revert in response to specific conditions, creating a mixed culture that is apparently necessary for proper colony formation. However, in contrast to ‘classical’ bistability, the stability, especially of explorers, appears to be very high (the transition rate is more than 24 h × 5 transfers).

In the soil, bacteria often cope with many fluctuations in the environment. This may be particularly true for swarm-ing (‘travelling’) bacteria, such as passage from watery to hard surfaces. The coexistence of explorers and builders in a planktonic population may be essential for a rapid trans-location and colonization in changing environment. One such example would be the ability of explorers to swarm through areas containing high levels of antibiotics, moving the colony through stressful niches, whereas the ability of P. vortex to revert to the mixed culture composition allows to recreate the colony in a newly colonized area.

The appearance of the different phenotypes of explorers when exposed to different concentrations of kanamycin may suggest a direct effect of kanamycin rather than intrinsic characteristics of explorers. The effect of non-lethal levels of antibiotics on bacterial motility has been previously reported (Kawamura-Sato et al., 2000; Park et al., 2008; Shen et al., 2008). However, this effect is usually limited to the exposure period and disappears after the removal of the antibiotics. The fact that the explorers’ phenotype is extremely stable may suggest that the explorers’ emergence is attributed to selection and enrichment by kanamycin. Nevertheless, this result does not disprove an additional effect of kanamycin on flagella genes.

The time it takes for bacteria to initiate swarming is called ‘swarm lag’ (Kearns, 2010). The increase in the number of flagella per cell in explorers may shorten the swarm lag in several ways: Hyperflagellated bacteria are prepared to swarm sooner after inoculation (reduced swarm lag) (Kearns and Losick, 2005; Kearns, 2010). The high number of flagella entangled with neighbouring cells may reduce the critical density of cells that is necessary to form raft that serves for cell translocation (Kirov et al., 2002; Ingham and Ben-Jacob, 2008; Kearns, 2010). This hypothesis may explain the previously observed phenomena of a short swarm lag in P. vortex (Ingham et al., 2011) and suggest that hyperflagellation can be an advantage for colony formation.

In this work, we have found that the fast-spreading explorers are hyperflagellated on one hand, and have reduced levels of cellular ATP on the other. It was previously shown that bacteria utilize PMF for flagella biosynthesis and motor function (Sowa and Berry, 2008; Minamino et al., 2011). Additionally, the flagellar switch–motor complex was found to consume ATP (Zarbiv et al., 2012). Hence, a possible explanation of ATP reduction could be in higher consumption of PMF and ATP by flagella biosynthesis and motor function, possibly as part of a diversion of a higher than normal fraction of the cells energy budget towards motility. The negative correlation between the expression levels of flagella genes and ATP levels in explorers supports this idea.

Reduction in ATP levels and a lower growth rate in liquid culture may also explain the increased resistance to kanamycin. There is some evidence that differentiation into
swarmers lowers metabolic activity unrelated to motility, and changes the physiology of the cell (Armitage, 1981; Kim and Surette, 2004). Explorers may be an example of swarmers that owe their adaptation to antibiotics to physiological changes (such as lower metabolic activity). This enhanced tolerance of explorers may explain the previously shown phenomenon of ‘pioneers’ and ‘secondary swarm fronts’ (highly tolerant subpopulations that cross antibiotic-containing areas) (Kim et al., 2003; Lai et al., 2009; Ingham et al., 2011). This phenomenon was
Fig. 7. Explorers isolated from different types of antibiotics. The mixed culture was grown for 18 h in liquid LB with various antibiotics (indicated on the left) at MICs. The expansion diameter, resistance to antibiotics, growth rate and growth yield of pre-exposed (explorers) and non-exposed culture were measured.

A–G. Expansion on LB agar. The pictures were taken after 24 h of growth on LB agar.
B–G. Colonies of explorers isolated from different types of antibiotics.
H–S. Disk diffusion assay.
H–M. Mixed cultures, N–S. explorers. Resistance to antibiotics was examined on LB plates using disk diffusion assay containing 50 μg kanamycin (H,N), 50 μg chloramphenicol (I,O), 5 μg spectinomycin (J,P), 5 μg ampicillin (K,Q), 0.1 μg rifampicin (L,R) and 5 μg novobiocin (M,S). The smaller the radius of inhibition zone, the higher the resistance level of the bacteria. We can see that explorers have a smaller inhibition zone, i.e. have a higher resistance. (I–VI) Growth curves of explorers (black) and mixed culture (grey). Explorers show a reduction in the growth rate at the mid (10–20 h) log phase (P < 0.002), and growth yield after 20 h (P < 0.008) in liquid in comparison to the mixed culture. Exceptional are the explorers isolated from ampicillin and rifampicin, (P < 0.03) and (P < 0.04), respectively, which shows similar growth kinetics to the mixed culture.

Experimental procedures

Culture

Liquid culture of P. vortex was in LB broth at 37°C with vigorous shaking at 220 rpm. Growth in liquid culture was measured at OD660 using EL808, BioTek spectrophotometer (Winooski, VT, USA). For colony growth, peptone plates contained 20 g l−1 peptone, 2.25% w/v agar, were poured with 12 ml of medium, and were allowed to solidify and dry until approximately 105/ml cells were inoculated into 96 wells containing 12 ml of medium, and were allowed to solidify and dry until 1 g of their original weight was lost. Peptone plates were incubated for 24–96 h at 30°C. For swarming assays and inhibition, Congo red (Sigma) was dissolved in distilled water, filter-sterilized and added to O.N. LB liquid culture diluted 1:100 at the shaking. Inoculations for swarming were made by depositing a 5 μl drop of an O.N. or pre-exposed culture grown in LB broth onto the centre of 1.5% w/v LB plate and incubated for 24 h at 37°C. For swarming inhibition, Congo red (Sigma) was dissolved in distilled water, filter-sterilized and added to LB or peptone agar just before pouring at a final concentration of 400 μg ml−1.

Disk diffusion assay

10⁵ cells from liquid cultures were swabbed uniformly across the LB agar. The plates were left to dry for 10 min. A filter paper disk with 50 μg kanamycin, 5 μg ampicillin, 50 μg chloramphenicol, 5 μg spectinomycin, 5 μg novobiocin and 0.1 μg rifampicin was placed at the centre of the plate. Plates were incubated O.N. at 37°C, and zones of inhibition were measured.

Image capture and light microscopy

Macrosopic images of the colonies were taken using digital camera (PowerShot A640, Canon, Melville, NY, USA). Colonies were stained with Brilliant Blue 0.1%, methanol 50% and acetic acid 10% to obtain higher contrast images. For microscopic images, pictures were taken using Olympus microscope under a total magnification of 200-fold (Olympus, Japan).

TEM

Actively swarming P. vortex were gently placed on the TEM grid by simply placing the grid against the surface of 1.5% LB plates. The grid with the collected sample was stained with uranyl acetate (negative staining) and observed in JEM 1200 EX electron microscope (JEOL Ltd., Akishima, Tokyo, Japan).

ATP measurements

Explorers isolated from 10, 20 and 40 μg ml−1 kanamycin were grown for 18 h in liquid medium. Bacteria from each culture were diluted to 10⁵ CFU/ml and treated according to manufacturer protocol (ATP lite, PerkinElmer, Waltham, MA, USA). The amount of emitted light (directly proportional to the ATP concentration) was measured with a luminometer. The ATP content (μM) of the cells was calculated using an ATP standard curve. ATP concentration was related to total cell extracted volume.

Real-time PCR

P. vortex RNA was used for reverse transcription RT-PCR Thermo Scientific kit; real-time PCR was performed using
Flagellin was extracted from *P. vortex* culture, grown O.N. in LB. Bacteria were centrifuged for 5 min at 2700 g, re-suspended in phosphate buffered saline and diluted to 10^9 CFU/ml. Flagella were removed by sonication in Ultra-re-suspended in phosphate buffered saline and diluted to 1010 CFU/ml. Flagella were removed by sonication in Ultra-cleaner (D80H, Taiwan) with heat treatment at 40°C for 10 min. Following sonication, bacteria were vigorously vortexed for 10 s and centrifuged at 20 800 g for 15 min. The supernatant containing flagellin was then collected and kept in 4°C.

**SDS-PAGE and Western blot**

The presence of flagellin was confirmed by 30 kDa band on SDS-PAGE 4–20% gradient gel (Bio-Rad Laboratories, Hercules, CA, USA). In addition, Western blot was performed using 1% w/v dried skim milk for blocking, 1:5000 IgG-HRP secondary anti-rabbit antibody and 1:1000 polyclonal anti-flagellin. Polyclonal anti-flagellin was raised in rabbits against the synthetic peptides: 1. RINRAADDAAGLAISEKMR: 2. GAVQVNRLEHTNVNLG. (Eurogentec, Belgium; http://www.eurogentec.com). For relative quantification of flagellin, ImageJ 1.46 software was used.

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**References**

Armitage, J.P. (1981) Changes in metabolic activity of *Proteus mirabilis* during swarming. *J Gen Microbiol* 125: 445–450.

Arnold, J.W., and Shimkets, L.J. (1988) Inhibition of cell-cell interactions in *Myxococcus xanthus* by Congo red. *J Bacteriol* 170: 5765–5770.

Balaban, N.Q., Merrin, J., Chait, R., Kowalcik, L., and Leibler, S. (2004) Bacterial persistence as a phenotypic switch. *Science* 305: 1622–1625.

Be’er, A., Florin, E.L., Fisher, C.R., Carolyn, R., Swinney, H.L., Harry, L., and Payne, S.M. (2011) Surviving bacterial sibling rivalry: inducible and reversible phenotypic switching in *Paenibacillus dendritiformis*. *mBio* 2: e00069–11.

Ben-Jacob, E. (1997) From snowflake formation to growth of bacterial colonies II: cooperative formation of complex colonial patterns. *Contemp Phys* 38: 205–241.

Ben-Jacob, E. (2003) Bacterial self-organization: co-enhancement of complexification and adaptability in a dynamic environment. *Phil Trans R Soc Lond A* 361: 1283–1312.

Ben-Jacob, E., and Cohen, I. (1997) Cooperative Formation of Bacterial Patterns. *Bacteria As Multicellular Organisms*. Shapiro, J.A., and Dworkin, M. New York, USA: Oxford University Press, pp. 394–416.

Ben-Jacob, E., and Levine, H. (2006) Self-engineering capabilities of bacteria. *J R Soc Interface* 3: 197–214.

Ben-Jacob, E., Cohen, I., Czirók, A., Vicsek, T., and Gutnick, D.L. (1997) Chemomodulation of cellular movement, collective formation of vortices by swarming bacteria, and colonial development. *Physica A Stat Mech Appl* 238: 181–197.

Ben-Jacob, E., Cohen, I., and Gutnick, D.L. (1998) Cooperative organization of bacterial colonies: from genotype to morphotype. *Annu Rev Microbiol* 52: 779–806.

Ben-Jacob, E., Cohen, I., Golding, I., Gutnick, D.L., Tcherpakov, M., Helbing, D., and Ron, I.G. (2000) Bacterial cooperative organization under antibiotic stress. *Physica A Stat Mech Appl* 282: 247–282.

Ben-Jacob, E., Aharonov, Y., and Shapira, Y. (2004) Bacteria harnessing complexity. *Biofilms* 1: 239–263.

Butler, M.T., Wang, Q., and Harshey, R.M. (2010) Cell density and motility protect swarming bacteria against antibiotics. *Proc Natl Acad Sci USA* 107: 3776–3781.

Cohen, I., Czirók, A., and Ben-Jacob, E. (1996) Chemotactic-based adaptive self-organization during colonial development. *Physica A Stat Mech Appl* 233: 678–698.

Darnton, N.C., Turner, L., Rojevsky, S., and Berg, H.C. (2010) Dynamics of bacterial swimming. *Biophys J* 98: 2082–2090.

Dubnau, D., and Losick, R. (2006) Bistability in bacteria. *Mol Microbiol* 61: 564–572.

Hamze, K., Autret, S., Hinc, K., Laalami, S., Julkowska, D., Briandet, R., et al. (2011) Single-cell analysis in situ in a *Bacillus subtilis* swarming community identifies distinct spatially separated subpopulations differentially expressing hag (flagellin), including specialized swarmers. *Microbiol Mol Biol Rev* 157: 2456–2469.

Harshey, R.M. (2003) Bacterial motility on a surface: many ways to a common goal. *Annu Rev Microbiol* 57: 249–273.

Ingham, C.J., and Ben-Jacob, E. (2008) Swarming and complex pattern formation in *Paenibacillus vortex* studied by imaging and tracking cells. *BMC Microbiol* 8: 36.

Ingham, C.J., Kalisman, O., Finkelstein, A., and Ben-Jacob, E. (2011) Mutually facilitated dispersal between the non-
motile fungus Aspergillus fumigatus and the swarming bacterium Paenibacillus vortex. *Proc Natl Acad Sci USA* 108: 19731–19736.

Kawamura-Sato, K., Inuma, Y., Hasegawa, T., Horii, T., Yamashino, T., and Ohta, M. (2000) Effect of subinhibitory concentrations of macrolides on expression of flagellin in *Pseudomonas aeruginosa* and *Proteus mirabilis*. *Antimicrob Agents Chemother* 44: 2869–2872.

Kears, D.B. (2010) A field guide to bacterial swarming motility. *Nat Rev Microbiol* 8: 634–644.

Kears, D.B., and Losick, R. (2005) Cell population heterogeneity during growth of *Bacillus subtilis*. *Genes Dev* 19: 3083–3094.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y., and Lewis, K. (2004) Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett* 230: 13–18.

Kim, W., and Surette, M.G. (2004) Metabolic differentiation in actively swarming Salmonella. *Mol Microbiol* 54: 702–714.

Kim, W., Killam, T., Sood, V., and Surette, M.G. (2003) Swarm-cell differentiation in salmonella enterica serovar typhimurium results in elevated resistance to multiple antibiotics. *J Bacteriol* 185: 3111–3117.

Kirov, S.M., Tassell, B.C., Semmler, A.B., O’Donovan, L.A., Rabaa, A.A., and Shaw, J.G. (2002) Lateral flagella and swarming motility in Aeromonas species. *J Bacteriol* 184: 547–555.

Lai, S., Tremblay, J., and Déziel, E. (2009) Swarming motility: a multicellular behaviour conferring antimicrobial resistance. *Environ Microbiol* 11: 126–136.

Lewis, K. (2010) Persister cells. *Annu Rev Microbiol* 64: 357–372.

Madi, A., Friedman, Y., Roth, D., Regev, T., Bransburg-Zabary, S., and Ben-Jacob, E. (2008) Genome holography: deciphering function-form motifs from gene expression data. *PLoS ONE* 3: e2708.

Matsuyma, T., and Matsuyma, M. (1993) Fractal morphogenesis by a bacterial cell population. *Crit Rev Microbiol* 19: 17–135.

Melter, O., and Radojevic, B. (2010) Small colony variants of *Staphylococcus aureus* – review. *Folia Microbiol (Praha)* 55: 548–558.

Minamino, T., Morimoto, Y.V., Hara, N., and Namba, K. (2011) An energy transduction mechanism used in bacterial flagellar type III protein export. *Nat Commun* 2: 475.

Park, S.Y., Kim, R., Ryu, C.M., Choi, S.K., Lee, C.H., Kim, J.G., and Park, S.H. (2008) Citrinin, a mycotoxin from *Penicillium citrinum*, plays a role in inducing motility of *Paenibacillus polymyxa*. *FEMS Microbiol Ecol* 65: 229–237.

Proctor, R.A., Kahl, B., von Eiff, C., Vaudaux, P.E., Lew, D.P., and Peters, G. (1998) *Staphylococcal* small colony variants have novel mechanisms for antibiotic resistance. *Clin Infect Dis* 27 (Suppl. 1): S68–S74.

Roth, D., Madi, A., Kenett, D.Y., and Ben-Jacob, E. (2011) Gene network holography of the soil bacterium *Bacillus subtilis*. In *Biocommunication in Soil Microorganisms*. Witzany, G. (ed.). Berlin, Heidelberg, Germany: Springer, pp. 255–281.

Shen, L., Shi, Y., Zhang, D., Wei, J., Surette, M.G., and Duan, K. (2008) Modulation of secreted virulence factor genes by subinhibitory concentrations of antibiotics in *Pseudomonas aeruginosa*. *J Microbiol* 46: 441–447.

Sirota-Madi, A., Olender, T., Helman, Y., Ingham, C., Brainis, I., Roth, D., *et al.* (2010) Genome sequence of the pattern forming *Paenibacillus vortex* bacterium reveals potential for thriving in complex environments. *BMC Genomics* 11: 710.

Sowa, Y., and Berry, R.M. (2008) Bacterial flagellar motor. *Q Rev Biophys* 41: 103–132.

Stewart, P.S., and Costerton, W.J. (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* 358: 135–138.

Velicer, G.J., Kroos, L., and Lenski, R.E. (1998) Loss of social behaviors by *Myxococcus xanthus* during evolution in an unstructured habitat. *Proc Natl Acad Sci USA* 95: 12376–12380.

Wilson, S.G., and Sanders, C.C. (1976) Selection and characterization of strains of *Staphylococcus aureus* displaying unusual resistance to aminoglycosides. *Antimicrob Agents Chemother* 10: 519–525.

Zarbib, G., Li, H., Wolf, A., Cecchini, G., Caplan, S.R., Sourij, V., and Eisenbach, M. (2012) Energy complexes are apparently associated with the switch-motor complex of bacterial flagella. *J Mol Biol* 416: 192–207.

Zhang, H.P., Be’er, A., Florin, E.L., and Swinney, H.L. (2010) Collective motion and density fluctuations in bacterial colonies. *Proc Natl Acad Sci USA* 107: 13626–13630.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Kinetics of colony development on peptone agar. *P. vortex* was grown for 18 h in liquid LB with and without 10 μg ml⁻¹ kanamycin. Cells were then inoculated on peptone plates, (20 g l⁻¹, 2.25% w/v agar) and grown in 30°C for 3 days (A). Pictures were taken after 24, 48 and 72 h. Scale bar = 5 mm. Colony diameter (branches width, mm), was measured after each time point (B).

**Fig. S2.** Builders’ susceptibility to kanamycin. *Builders* enriched culture, explorers and mixed culture were examined for resistance to kanamycin using disk diffusion assay. The bars represent the averaged values of inhibition radius. A significant difference is found between builders’ and explorers’ inhibition radius with *P* < 8.6x10⁻⁰⁷. Pictures on top of each bar represent the inhibition zone of each morphotype respectively. Scale bar – 5 mm.

**Fig. S3.** Identification of explorers. Cells isolated from 10, 20 and 40 μg ml⁻¹ kanamycin were spread over LB agar. Microcolonies were picked, re-inoculated on LB agar, and the expansion diameter was examined. An example of swarming assay of four isolated samples after 24 h of incubation on LB plates. The expansion diameter (mm) presented in Table S1A.

**Fig. S4.** Growth of builders’ enriched culture in the presence of kanamycin. *Builders* enriched culture (blue) obtained after X 4 transfers at the early log phase, were grown in liquid LB for 8 h (A), 12 h (B) and 24 h (C). After each time point, samples were spread over LB agar, then microcolonies were immediately picked and added into liquid LB with (dashed line) and without (smooth line) 20 μg ml⁻¹ kanamycin, and

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growth curves were examined. Mixed culture grown to the same time points were taken as a control (black). (A) After 8 h, the builders did not grow in the presence of kanamycin compared with mixed culture. (B and C), After 12 h and 24 h, the culture had the same growth kinetics as the mixed culture, suggesting builders are stable up to 8 h but revert to the mixed culture composition by 12 h.

**Fig. S5.** Gene expression of *P. vortex* under kanamycin exposure. Gene expression profile of *P. vortex* culture after exposure to kanamycin. Normalized log signals (natural log, based on ‘e’) of ‘control’ and ‘kanamycin’ duplicates were averaged, and the difference between the averages was calculated. Upregulated genes are all the genes whose expression ratio is above 1.5 (log C > 0.4). The genes were filtered based on the presence of the kanamycin-induced upregulation and divided according to functional categories annotated by TIGR. The bars represent the percentage of upregulated genes from the total number of genes at each category.

**Fig. S6.** Holographic network of *P. vortex* flagella genes. Holographic network of flagella genes after 18 h of exposure to antibiotics. The correlation lines connecting the genes were set by correlation threshold greater than 0.7 and smaller than −0.4. The matrices sorted by dendrogram algorithm.

**Fig. S7.** Increase of flagella filaments number in explorers isolated from 40 μg ml⁻¹ kanamycin (A,B) or 15.6 μg ml⁻¹ chloramphenicol (C,D). A,C Mixed culture. B,D explorers. Magnification of X 50 K. Scale bar = 500 nm.

**Fig. S8.** RAPD-PCR of explorers. Columns 1 – 100bp Marker (Tal Ron). Columns 2,5 – Explorers. Columns 3,6 – *B. subtilis* 168 (negative control). Columns 4,7 – *P. vortex* (mixed culture). Primers for amplification – 16SrRNA (2–4), SPOVFA (5–7). Amplification profiles of explorers and *P. vortex* were similar under both primers in contrast to *B. subtilis* 168, indicating that explorers and *P. vortex* are the same species.

**Table S1.** Detection of explorers in the culture. (A) Colony expansion diameter (mm) of isolated explorers (Fig. S3). (B) The absorbance at O.D₆₅₀ of the same isolates, after 20 h of growth. Isolates that have shown 10% increase in colony diameter (A) and over 10% decrease in the absorbance after 20 h (B) were defined as explorer. The values of each isolate were compared with the average value of the control (bold). Isolates with a colony diameter value > 7.01 and O.D < 0.207 were identified as explorers (marked in grey). Evaluation of explorers' percentage was determined by division of the number of samples identified as explorers by the total number of samples.

**Table S2.** Expression of flagella and chemotaxis genes under exposure to kanamycin. All genes represented in the table have expression ratio above 1.5 (log C > 0.4). Genes' IDs were represented by the Query Locus Tag, published in NCBI's GEO [GSE35271].

**Table S3.** Primers for flagella genes, used for RT-PCR. Genes' IDs were represented by the Query Locus Tag, published in NCBI's GEO [GSE35271].

**Table S4.** Annotation of hypothetical genes.

**Table S5.** Emergence of explorers under different antibiotics.