Molecular Microbiology (1998) **27**(4), 687–703

# Classification and genetic characterization of pattern-forming *Bacilli*

**Rivka Rudner,* Oksana Martsinkevich, Wendy Leung and Erich D. Jarvis†**
*Department of Biological Sciences, Hunter College of the City University of New York, 695 Park Avenue, New York, NY 10021, USA.*

## Summary

**One of the more natural but less commonly studied forms of colonial bacterial growth is pattern formation. This type of growth is characterized by bacterial populations behaving in an organized manner to generate readily identifiable geometric and predictable morphologies on solid and semi-solid surfaces. In our first attempt to study the molecular basis of pattern formation in *Bacillus subtilis*, we stumbled upon an enigma: some strains used to describe pattern formation in *B. subtilis* did not have the phenotypic or genotypic characteristics of *B. subtilis*. In this report, we show that these strains are actually not *B. subtilis*, but belong to a different class of *Bacilli*, group I. We show further that commonly used laboratory strains of *B. subtilis* can co-exist as mixed cultures with group I *Bacilli*, and that the latter go unnoticed when grown on frequently used laboratory substrates. However, when *B. subtilis* is grown under more stringent semiarid conditions, members of group I emerge in the form of complex patterns. When *B. subtilis* is grown under less stringent and more motile conditions, *B. subtilis* forms its own pattern, and members of group I remain unnoticed. These findings have led us to revise some of the mechanistic and evolutionary hypotheses that have been proposed to explain pattern growth in *Bacilli*.**

## Introduction

Traditionally, microbiologists are accustomed to visualizing bacterial populations on agar surfaces in the form of round compact colonies. However, it has long been known (Gause, 1939; Winogradsky, 1949; Costerton *et al*., 1987; Winpenny, 1992) that, in their natural environment, bacteria grow more commonly as multicellular organized populations forming complex patterns (for review, see Shapiro, 1995). For *Bacillus subtilis*, various conditions have been shown to promote patterned growth, ranging from nutrient-poor hard agar surfaces (Fujikawa and Matsushita, 1989; Ben-Jacob *et al*., 1992; 1994a,b; Fujikawa, 1994) to nutrient-rich soft agar (Salhi and Mendelson, 1993; Mendelson and Salhi, 1996). Likewise, various and sometimes contradictory hypotheses have been proposed to explain why pattern formation occurs, i.e. adaptive morphogenesis, the need for a colony to alter its shape to survive in adverse environments, (Ben-Jacob *et al*., 1994b; 1995a; Shapiro, 1995) or humidity response, the ability of the bacteria to respond to other environmental factors, such as local humidity (Mendelson and Salhi, 1996), regardless of environmental adversity.

The term 'morphotype' has been coined to refer to the distinctive reproducible geometrical character of a given bacterial pattern (Ben-Jacob *et al*., 1995a). At present, at least five morphotypes for *B. subtilis* have been described under the nutrient-poor hard agar conditions (Ben-Jacob *et al*., 1995a). These are: (i) the common compact round colonial growth with rough edges (*B*); (ii) tree branches with tip-splitting growth (*T*); (iii) chiral growth with branches that have the same handedness (*C*); (iv) vortex growth with branches led by bacteria droplets that spin around a common centre (*V*); and (v) spiral vortex (*SV*). The *T* and *C* morphotypes were found to interconvert easily, where *C* is the preferred colony form grown on low agar concentrations and *T* on harder agar (Ben-Jacob *et al*., 1992; 1995b). When forming the *C* morphotype, the bacteria are longer than the *T* type by a factor of five (Ben-Jacob *et al*., 1994c). On hard surfaces, the *C* cells move forwards and backwards, while the *T* cells move randomly (Ben-Jacob *et al*., 1994a,c). The *V* and *SV* cells align collectively and spin with the same velocity clockwise or counterclockwise around a leading droplet made of a wetting fluid (Ben-Jacob *et al*., 1997). Other *B. subtilis* morphotypes have been found to arise under nutrient-rich soft agar conditions (Matsuyama and Matsushita, 1993; Mendelson and Salhi, 1996), but no names have been given to these shapes. In these studies, variations in agar wetness and nutrient levels were found to govern whether or not *B. subtilis* produced patterns. In general, when forming a pattern, the bacteria move by various translocations within a wetting fluid that they either excrete or process from the agar substrate (Henrichsen,

Received 4 September, 1997; revised 5 November, 1997; accepted 13 November, 1997. †Present address: The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA. *For correspondence. E-mail rudner@genectr.hunter.cuny.edu; Tel. (212) 772 5231; Fax (212) 772 5227.

1972; Ben-Jacob *et al*., 1992; Matsuyama and Matsushita, 1993; Fujikawa, 1994).

To characterize the *Bacilli* that form these fascinating patterns further, we undertook a molecular genetic approach involving comparing bacteria in their round, compact colonial state with their complex patterned state under different nutrient conditions. Using phenotypic analyses, Southern hybridizations and 16S ribosomal RNA comparisons, we show that the bacteria that have been reported to form the *C*, *T*, *V* and *SV* morphotypes are not *B. subtilis*, a group II bacillus, but instead belong to group I *Bacilli*, whose members include *B. alvei*, *B. macerans*, *B. popillae* and *B. circulans* (as grouped by Piest, 1993, his Table 1). Members of group I *Bacilli* are able to co-exist at a very low density with group II *Bacilli* and have been erroneously mistaken for *B. subtilis* for the last 10 years that pattern formation in this species has been studied. *B. subtilis* and other members of group II form patterns under nutrient-rich soft agar conditions, whereas group I *Bacilli* form patterns on both nutrient-poor hard agar and nutrient-rich soft agar substrates. The approach we used and the resulting classifications will help in discovering which of the various hypotheses that have been put forward to explain pattern growth are valid and will provide the necessary information towards an understanding of the molecular processes that govern pattern formation in *Bacilli*.

## Results

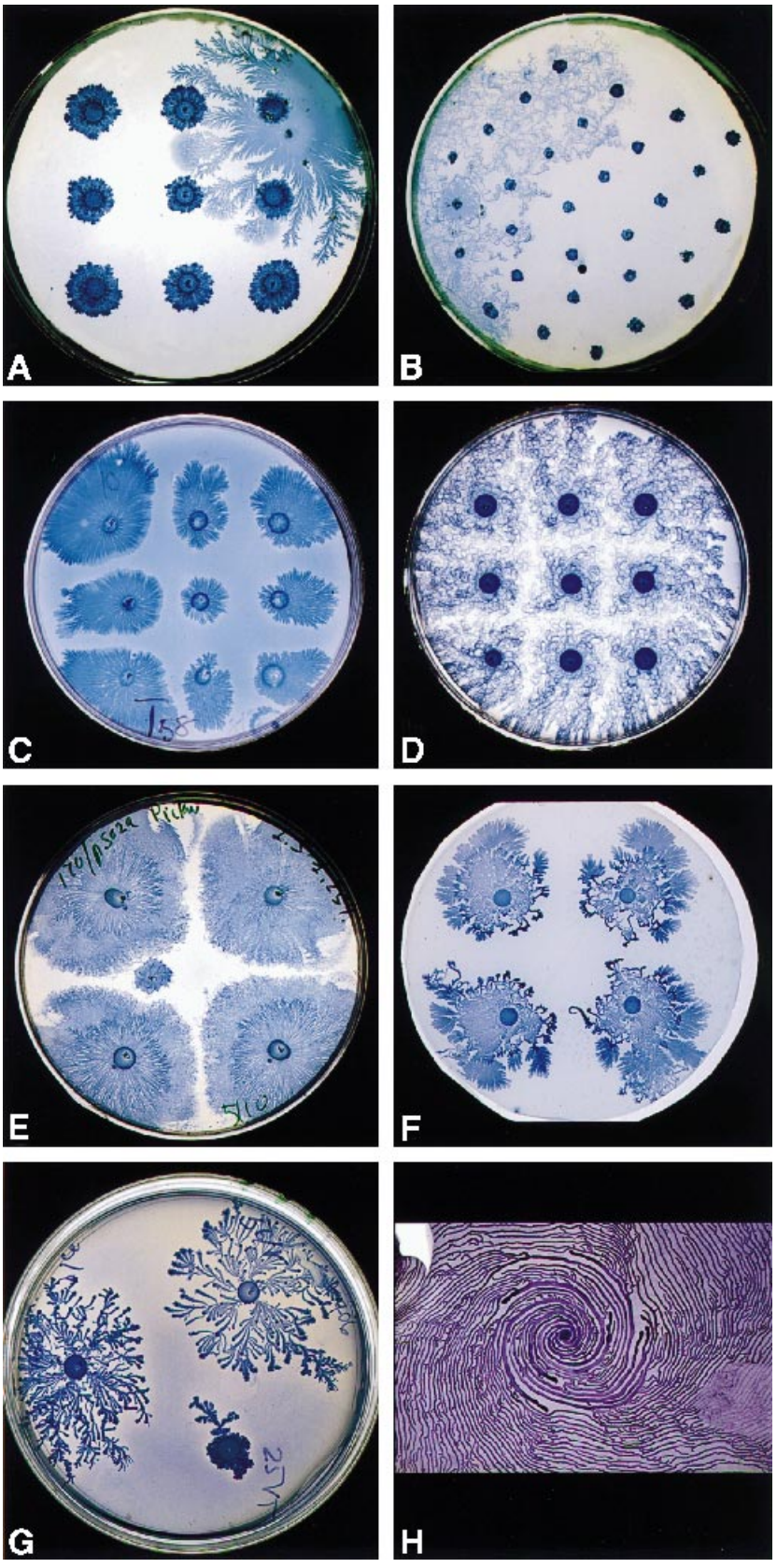
### *Generation of two kinds of pattern formers*

In order to reconcile the various hypotheses that have been proposed to explain how and why pattern formation occurs in *B. subtilis*, we made parallel comparisons of *B. subtilis* suspensions grown under the various nutrient/agar conditions that were reported to elicit patterned growth (Ben-Jacob *et al*., 1992; Matsuyama and Matsushita, 1993; Mendelson and Salhi, 1996). We chose to compare two opposite conditions, low-nutrient hard agar and high-nutrient soft agar in reciprocally inoculated cell suspensions.

When cell suspensions ($1–5 \times 10^5$) originating from single colonies of established *B. subtilis* strains were point inoculated under the first condition, nutrient-poor (0.1–1% peptone) thin hard surfaces (1.25–2.5% agar), the majority of the bacterial colonies remained round and relatively compact as a *B* morphotype but with some minimum branching (Fig. 1A and B). However, after long incubation periods of 7–30 days, various highly branched colonial patterns resembling *T*, *C* and other morphotypes developed from some of the colonies. As shown in Fig. 1A and B, a *T* or a *C* pattern was generated on plates containing either nine or 30 colonies, respectively, from many (30–60) similarly inoculated plates. The *T* pattern had characteristic branching, spreading out from the centre of the colony in tip-splitting growth (Fig. 1A); the *C* pattern had chiral twisting with the same handedness towards the right (Fig. 1B). The frequency of pattern formation varied with the strains used, from none to multiple occurrences (see Table 3). When cells taken from the branched tips of these patterns were grown in liquid rich medium and then spotted onto another nutrient-poor hard agar plate, pattern formation occurred much faster (24–48 h) and at a frequency of 100% (see Fig. 1C for the *T* and Fig. 1D for the *C*). As reported earlier (Ben-Jacob *et al*., 1995b), we generated interconversions between *T* and *C* patterns by making small changes in agar concentration (Fig. 1E and F). The vortex (*V*) and spiral vortex (*SV*) shown in Fig. 1G and H, respectively, did not emerge among our inoculates, even though we tried some of the stringent requirements for their emergence, such as lower temperatures (Ben-Jacob *et al*., 1997). Thus, for these two morphologies, we used the *V*s and *SV*s generated in Ben-Jacob's laboratory. None of the described *T*, *C*, *V* or *SV* morphotypes was found to revert back to the *B* type.

When the initial cell suspension described above was point inoculated, in parallel, on thick nutrient-rich TB or VT–0.6% agar-Xgal plates, pattern formation occurred much faster and at a higher frequency than it did on nutrient-poor hard agar. As shown in the examples, on two separate plates, four out of nine and six out of nine colonies produced patterns upon repeated platings (Fig. 2A and B). We name this pattern as the deep-branching (*DB*) morphotype, because it consists of finger-like

**Fig. 1.** The emergence of *T* and *C* patterns from *B. subtilis* suspensions grown on nutrient-poor hard agar and representative patterns developed by established *T*, *C*, *V* and *SV* morphotypes.
A. Nine point inoculations of a suspension of strain IS58 on a $2\,g^{-1}$ peptone, 1.5% agar incubated for 7 days at 37°C resulted in the emergence of a single *T* pattern.
B. Thirty point inoculations of strain IS56/*pPW810-veg* on a $1.25\,g^{-1}$ peptone, 1.25% agar incubated for 9 days at 37°C resulted in the emergence of a *C* pattern.
C and D. Cells taken from the branched tips of the patterns in A and B, respectively, grown in liquid cultures, inoculated on the same type of plate and incubated at 37°C for 24–48 h.
E and F. *T*→*C* and *C*→*T* morphotype interconversions grown on $1.5\,g\,l^{-1}$ peptone, 1.25% agar and $1.25\,g\,l^{-1}$ peptone, 1.75% agar respectively. Plates were incubated at 37°C for 72–96 h.
G and H. *V* and *SV* morphotypes (Ben-Jacob *et al*., 1997), generated from strains SB25 and IS56/*pPW810-veg*, grown on $4\,g\,l^{-1}$ peptone plus 2.5% agar and $2\,g\,l^{-1}$ peptone plus 1.5% agar, respectively, incubated at 30°C for 72 h. All plates were stained with brilliant blue as described in *Experimental procedures*.


A
B
C
D
E
F
G
H

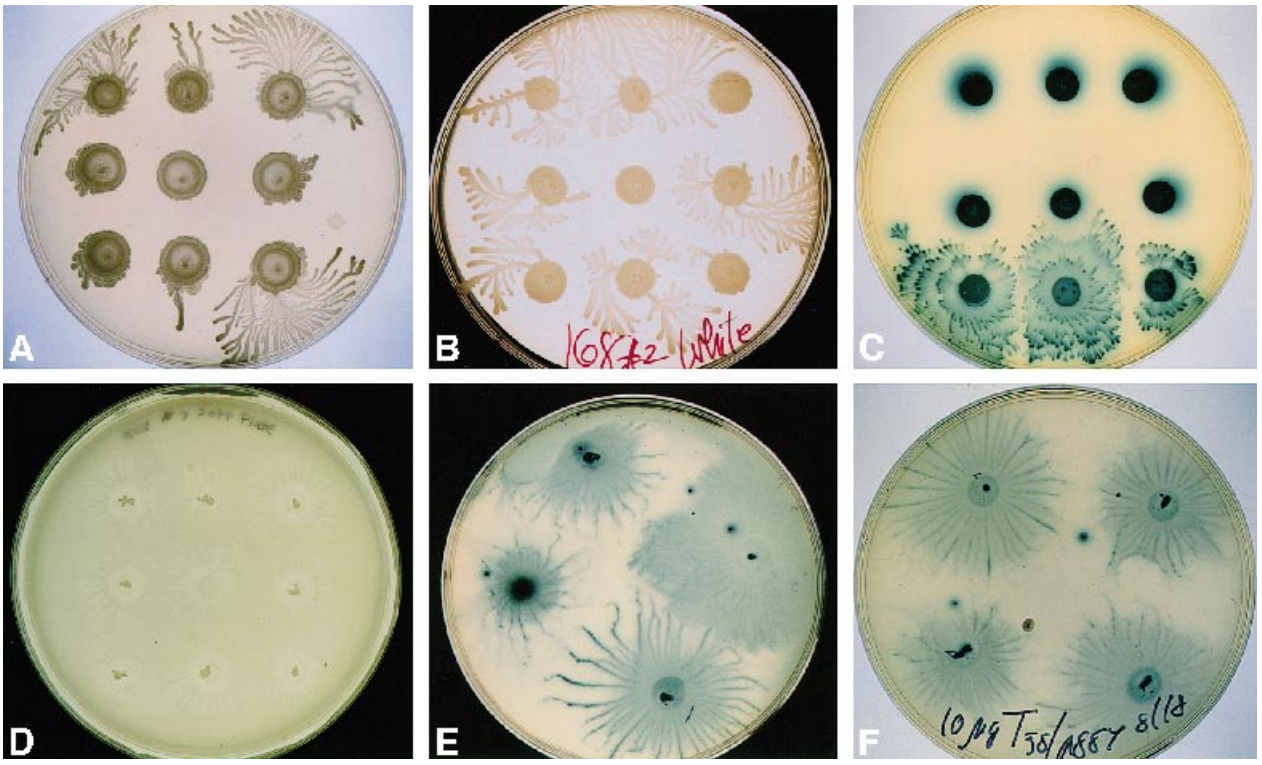



**Fig. 2.** Patterns of *B. subtilis, T*, *C* and *V* morphotypes on nutrient-rich, soft agar plates.
A. Strain BD170 point inoculated nine times onto TB–0.6% agar containing 160 μg ml$^{-1}$ Xgal.
B. Strain 168T grown on VT–0.6% agar containing 160 μg ml$^{-1}$ Xgal.
C. Three different integrant strains containing plasmid pPW810-*veg* (top row BD170, middle row SB25, bottom row BD79) grown on a similar VT plate containing 10 μg ml$^{-1}$ chloramphenicol.
D. Strain IS58 grown on a TB plate inoculated 48 h after preparation.
E. Morphotypes clockwise from upper left, *T*, *V*, *T* and *C* grown on a VT plate.
F. *T* generated from IS56/pER102-*leu*, grown on a VT plate.

projections that expand outwards from the centre of the colony. When cells taken from the branched tips of these patterns were grown in liquid-rich medium and then inoculated onto another nutrient-rich soft agar plate, most grew again with the same pattern. Some of the reinoculations 'reverted' back to compact growth (not shown). This is unlike the morphotypes generated on nutrient-poor hard agar that did not revert back to compact growth. The patterns generated varied among strains of *B. subtilis* and in response to the agar wetness. For example, of three *B. subtilis* strains (BD170, SB25 and BD79) containing an integrated *lacZ* fusion gene (pPW810-*veg*) at the *amyE* locus, plated on the same plate and on a given day, only three out of three of the latter strain produced a pattern (Fig. 2C). We define this pattern here as layered radial (*LR*) morphology, exhibiting maximal reporter gene expression at the finger tips, and it is similar to that described by Mendelson and Salhi (1996). On other occasions, the same strains with or without the *lacZ* fusion gene yielded patterns on similar, but not from the same batch of, plates (Fig. 2A). This could be explained by variation in the plate wetness. At higher wetness, i.e. plates 48 h after preparation, the colonies swarmed over the entire surface, and the patterns were hard to discern after 24 h incubation at 37°C (Fig. 2D). In contrast, when the plates were drier (3–5 days after preparation), patterns emerged slowly, mostly from the colonies at the plate periphery, with the pattern also restricted to the periphery (Fig. 2A and B).

When suspensions of the purified *T*, *C* and *V* morphotypes (Fig. 1C, D and G) generated on the nutrient-poor hard agar surfaces were point inoculated on the nutrient-rich soft agar substrate, their defined morphologies of tip-splitting (*T*), chiral (*C*) and vortex (*V*) growth were not reproduced. Instead, all gave rise to blue thorny spiky morphologies in a 24–48 h incubation period on the TB–0.6% agar-Xgal plates (Fig. 2E and F). Conversely, when suspensions of the *DB* and *LR* morphotypes initially generated on nutrient-rich soft agar plates were then point inoculated onto the nutrient-poor hard agar surfaces, the vast majority did not form a pattern and remained compact, as seen for the initial suspension. Those few that showed a pattern had the *T* and *C* morphologies.

*Genus identification of the various morphotypes*

Owing to the low frequency of occurrence of the *T*, *C* and *V* morphotypes and the stable inheritance of their pattern property once it is formed, we considered what others had proposed (Ben-Jacob *et al*., 1995a), that the morphotypes resulted from an adaptive mechanism involving genome reorganization. To address this issue, we analysed several genes by Southern blot hybridizations using two single-copy *B. subtilis* markers, *leu* and *thr*, and one multicopy highly conserved gene, 23S-5S rDNA. The *leu* and *thr* probes failed to hybridize to *Eco*RI-digested DNA from *C*, *T* and *V* morphotypes (Fig. 3B). DNA isolated from the compact *B* type from nutrient-poor hard agar (Fig. 1A) and from those that did or did not form a pattern on the nutrient-rich soft agar (Fig. 2A) revealed the appropriate size bands of 6.0 kb *leu* and 1.5 kb *thr* (Fig. 3B; Rapoport *et al*., 1978; Gottlieb, 1984).

The *B. subtilis* ribosomal 23S-5S probe hybridized to all morphotype DNAs with similar intensity at a stringency of 60°C (Fig. 3A). However, the blots revealed extensive polymorphism. The sizes of the *T*, *C* and *V rrn* homologues did not coincide with those traditionally described for *B. subtilis* (Fig. 3A; Rudner *et al*., 1993). *Eco*RI digests of *B. subtilis* DNA are known to reveal nine or 10 *rrn* homologues with the 2.9 kb *rrnH*-5′-*rrnG* representing the variable homologue (Fig. 3A; Widom *et al*., 1988; Rudner *et al*., 1993). The *C* and *T* band patterns were identical and exhibited 11–12 *rrn* fragments, none in alignment with the *B. subtilis* fragments. Similarly, the *V rrn* fragments were different from *B* and the other morphotypes. In contrast, the band pattern of morphotypes generated on the nutrient-rich soft agar condition was the same as *B. subtilis* (Fig. 3A).

The lack of ribosomal rDNA fragment size similarity between the *C*, *T* and *V* morphotypes and the original *B. subtilis* suspensions could be caused by rearrangements that occurred outside the ribosomal genes during pattern formation leading to variations in the location of *Eco*RI sites, or it could be caused by a completely different *rrn* gene, i.e. another species. We, therefore, amplified the 16S DNA of each morphotype by polymerase chain reaction (PCR) using conserved primers according to Suzuki and Yamasato (1994). All DNA templates generated the same size 1.5 kb fragment (Fig. 4B), which we then purified and sequenced. The entire sequences have been submitted to the GenBank database (nos. AFO39408 and AFO39409 for C/T and V respectively). It became apparent that the *T*, *C* and *V* morphotypes were not *B. subtilis*, as seen by differences in sequences (Fig. 4A). The DNA from *T*, *C* and *V* has only 88% identity with *B. subtilis*. The 16S sequences of *T* and *C* were identical to each other and different from *V* (Fig. 4A). Comparisons with GenBank revealed a 95% and 94% identity to *Bacillus alvei* and

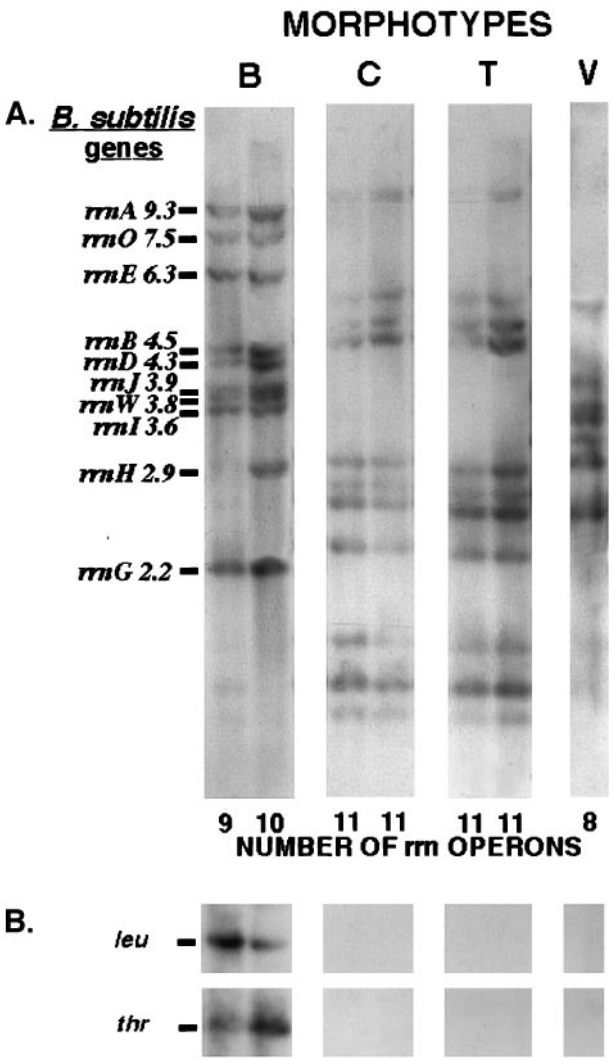



**Fig. 3.** Southern blots of *Eco*RI-digested chromosomal DNAs from normal *B. subtilis* and *T*, *C* and *V* morphotypes hybridized with *rrn*, *leu* and *thr* probes.
A. From right to left, strains BD79 and 168T containing nine and 10 *rrn* operons, respectively, representing the restriction profiles of those bacteria which did not form a pattern on nutrient-poor hard agar but did form patterns sometimes on nutrient-rich soft agar; the two *T*s emerged from 168T and BD79, the two *C*s from IS58 and IS56 and the *V* from IS56/pPW810-*veg*. The DNAs were digested with *Eco*RI, electrophoresed on 0.8% agarose and, after transfer, probed with a labelled 23S-5S fragment.
B. The same DNAs as above probed with *leu* and *thr* probes as described in *Experimental procedures*.

*Bacillus macerans* for *C*/*T* and *V* morphotypes respectively. At least two variable regions were noticed at nucleotides 170–220 and 454–481, in which *C*/*T* was similar to *B. alvei* and *V* to *B. macerans*, but both were dissimilar to *B. subtilis*. Those bacteria that (i) did or (ii) did not form a pattern on nutrient-rich soft agar; and (iii) those that did not form a pattern on nutrient-poor hard agar were identical to *B. subtilis*. Conversely, *C*/*T* and *V* morphotypes grown

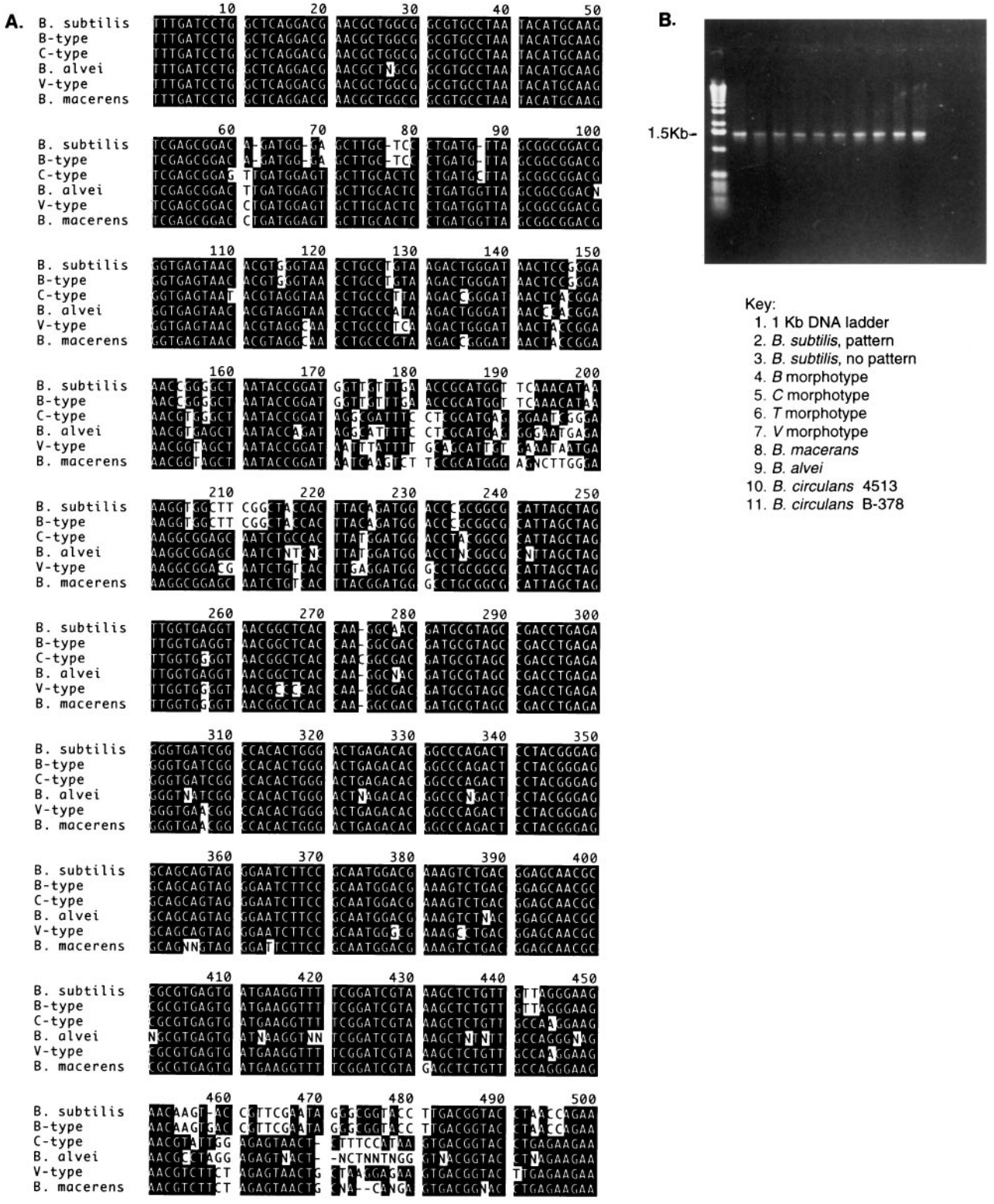



**Fig. 4.** The sequences of the first 500 bp of 16S rDNA derived from the 1.5 kb amplified fragments of *B. subtilis*, *C*/*T* and *V* morphotypes.
A. The sequences were aligned to the *B. subtilis* 16S *rrnA* gene (Ogasawara *et al.*, 1983) obtained from GenBank (top line), as were the *B. alvei* (Ash *et al.*, 1991) and the *B. macerans* (O. Shida, unpublished, 1995).
B. Gel electrophoresis of PCR products obtained by amplification of different *Bacilli* DNAs using conserved primers that align to each end of the 1.5 kb 16S DNA gene. Lanes 2–4 are *B. subtilis* strains 1A718, 168T/pPW810-*veg* and BD79 respectively; lanes 5 and 6 are *C* and *T* that emerged from strain SB25; lane 7 is an *SV* that emerged from strain IS56/pPW810-*veg*; lanes 8 and 9 are strains of *B. macerans* B-4267 and *B. alvei* B-383; the last two strains were *B. circulans* as indicated (see Table 3 for strain description).

**Table 1.** Physiological properties of *Bacilli* morphotypes.

| Morphotype or strain[a] | Growth requirement | Generation time (min) | Drug resistance[b] | β-Galactosidase activity[c] | Pattern ability: Hard agar | Pattern ability: Soft agar |
|---|---|---|---|---|---|---|
| *B* | None | 55 | S | – | – | – |
| *DB* and *LR* | None | 55 | S | – | – | + |
| *T* | Vitamins | 90 | R | + | + | + |
| *C* | Vitamins | 125 | R | + | + | + |
| *V* | Vitamins | 145 | r | + | + | + |
| *SV* | Vitamins | 150 | r | + | + | + |
| *B. circulans* | Leu, Thr | 62 | R | + | + | + |
| *B. cereus* | Vitamins | 64 | R | + | + | + |
| *B. macerans* | ND | 98 | R | + | + | + |
| *B. alvei* | ND | 108 | r | ± | + | + |
| B. *popillae* | ND | 95 | r | ± | + | + |

**a.** *B*, *DB* and *LR* represent *B. subtilis* isolated from nutrient-rich low agar that did not and did form patterns (see Fig. 2A–C). *B. circulans* strain ATTC 4513; *B. cereus* given to us as a *B. macerans* strain BKM-51; *B. macerans* strain NRRL B-4267; *B. alvei* strain NRRL B-383; *B. popillae* strain NRRL B2309; see Table 3 for origin of strains.
**b.** Drugs tested were Cm, Erm, Km, Sm, Spc, Am, Tc. All species were $Rif^S$. R, growth on drug plate in 24 h; r, slow growth.
**c.** β-Galactosidase-like activity on Xgal plates; +, dark blue after 24 h; ±, pale blue.
ND, not determined.

on nutrient-rich soft agar revealed the same sequence identity to *B. alvei* and *B. macerans* respectively. Both *B. alvei* and *B. macerans* belong to a separate group of *Bacilli*, known as group I, whereas *B. subtilis* belongs to group II (Piest, 1993). Thus, the ribosomal RNA genes and their surrounding sequences of the *C*, *T* and *V* morphotypes strongly suggest that they belong to group I *Bacilli*.

### *Genetic and physiological characterization of group I morphotypes*

To verify that the *C*, *T* and *V* morphotypes actually belonged to group I *Bacilli*, we performed phenotypic analysis of these strains along with known group I *Bacilli*. We chose *B. macerans* and *B. alvei* because of their high homology to the *T*, *C* and *V* morphotypes, *B. popillae* for similar spore coat morphologies (see below; Aronson and Fitz-James, 1976) and *B. circulans* for its known circular spreading on hard agar surfaces and, thus, its name (Ford, 1916; Smith and Clark, 1938). These results are summarized in Table 1 and outlined below.

*Growth requirements*. The *T*, *C*, *V* and *SV* morphotypes did not grow in liquid minimal medium. However, they did grow when the medium was enriched with either vitamin-free casamino acids and yeast extract (enMM; described in *Experimental procedures*) or a mixture of six vitamins (thiamine HCl, nicotinic acid, riboflavin, p-aminobenzoic acid, pyridoxine and biotin; Table 1). Similarly, the group I *Bacilli* we tested also exhibited these additional growth requirements. Thus, as has been documented for group I *Bacilli* (Piest, 1993), the *T*, *C*, *V* and *SV* morphotypes were also auxotrophs.

*Growth rates–generation times*. When grown in complex medium (LB or VY), the *T*, *C*, *V* and *SV* morphotypes showed considerable clumping after overnight incubations at 37°C. The clumping was less pronounced in enMM or at 30°C in complex medium. Their population doubling times in enMM were slower, ranging from 90 to 150 min compared with 55 min for the *B. subtilis* cultures (Table 1). Similarly, group I *Bacilli* exhibited clumping after growth in complex medium and doubling times in enMM of 62–108 min (Table 1).

*Drug resistance*. Group I *Bacilli* are known to have a general resistance to antibiotics (Piest, 1993). Likewise, we found the *T*, *C*, *V* and *SV* morphotypes were resistant to the following antibiotics: chloramphenicol (Cm), lincomycin-erythromycin (Erm), kanamycin (Km), ampicillin (Am), tetracycline (Tc), spectinomycin (Sp) and streptomycin (Sm); however, they were not resistant to rifampin (Rif; Table 1). These resistances were exhibited only on agar surfaces but not during growth in liquid media. On the various antibiotic plates, both our morphotypes and the group I *Bacilli* exhibited swarming associated with the excretion of wetting fluids and the production of a foul odour. None of the *B. subtilis* strains we used showed these drug-resistant properties or the emission of the fluids and odour.

*β-Galactosidase activity*. A most surprising phenotype was noted: the induction of a β-galactosidase-like activity produced a blue colour on plates containing Xgal for the *T*, *C* and *V* morphotypes (Fig. 2E and F) and the four group I *Bacillus* species but *B. subtilis* remained white (Fig. 2A and B). Among group I *Bacilli*, *B. alvei* strain

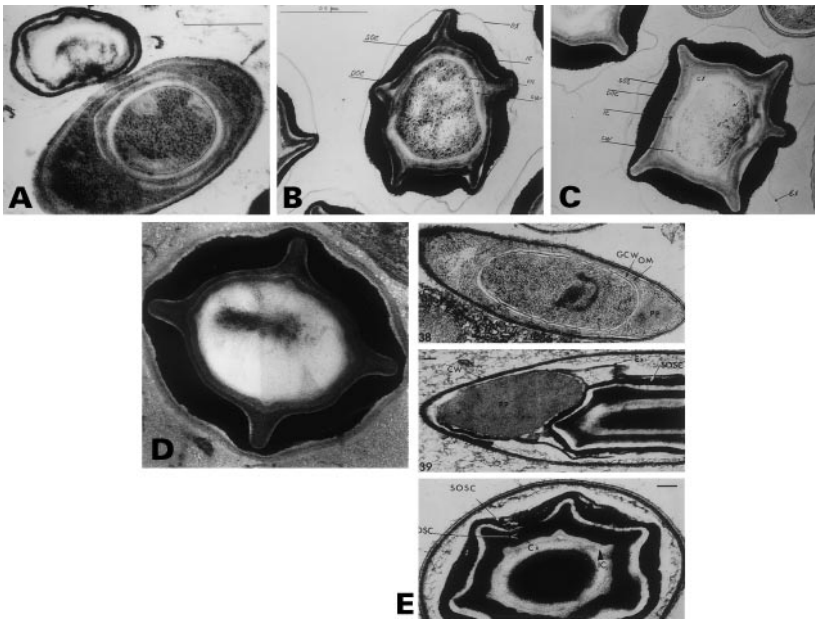



**Fig. 5.** Electron micrographs of thin spore sections of normal *B. subtilis C*, *T* and *V* morphotypes, and *B. popillae*.
A. The *B* morphotype (168T/pPW810-*veg*) at ×75 000. Upper left shows the mature spore.
B. The *C* morphotype at ×82 000.
C. The *T* morphotype at ×84 780.
D. The *V* morphotype at ×104 000.
E. *B. popillae* in various stages of sporulation taken from Aronson and Fitz-James (1976) in their Figs 38, 39 and 40 at ×59 500, ×59 000 and ×102,000 respectively; mature spore shown at the bottom. Spore components described in B, C and E designate the following: coat wall (CW); cortex (Cx); dense outer coat (DOC), exosporium (EX); germ cell wall (GCW); lamellar or multilayer inner coat (IC); inner membrane (IM); nucleoid (N); outer forespore membrane (OM); parasporal protein (PP); ribosomes (R); spongy outer (spore) coat (SOC/SOSC).

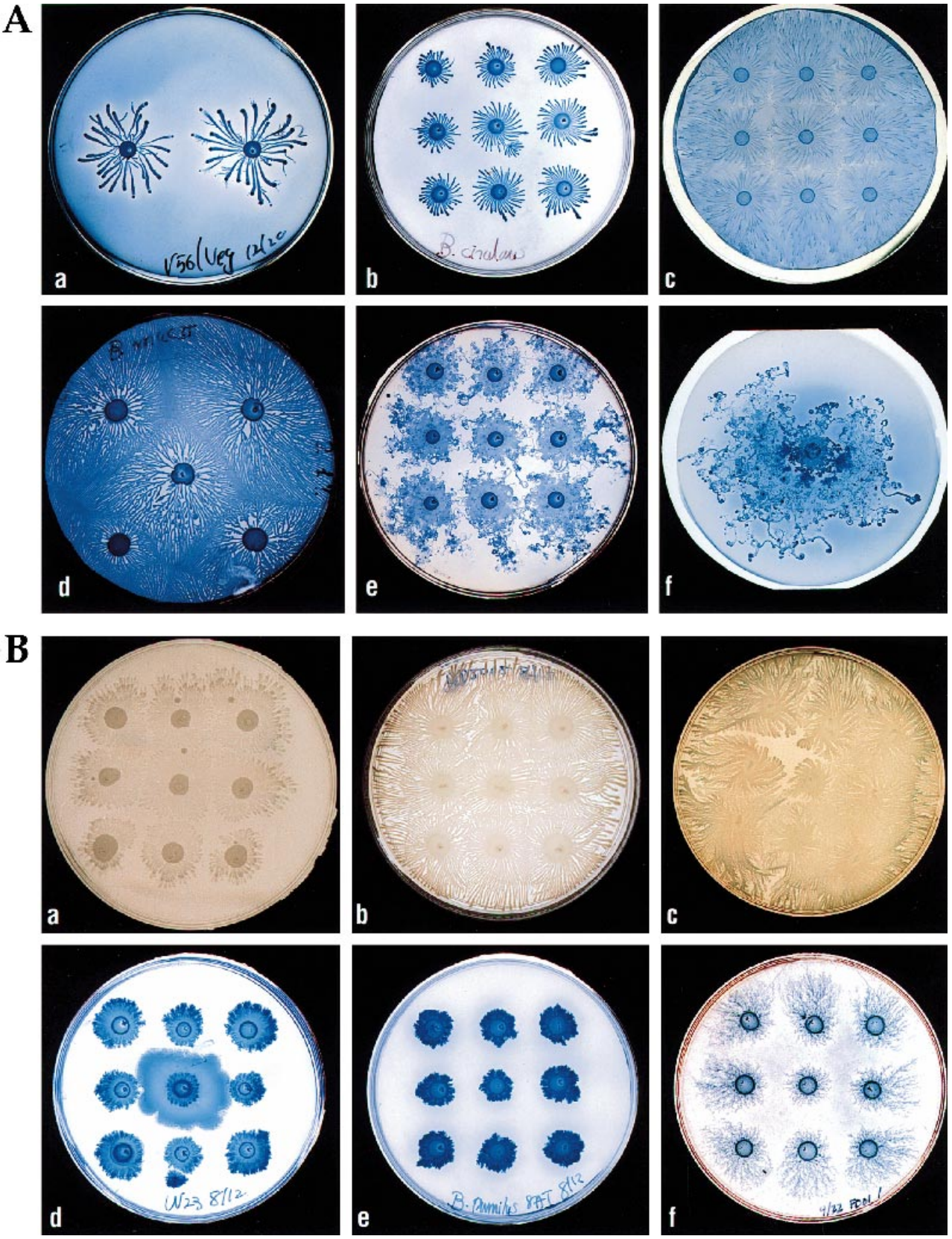
B-383 and *B. popillae* B-2309 showed a delayed appearance of a pale blue colour (2–3 days; Table 1). In contrast, after long incubation periods (7–20 days), *B. subtilis* showed weak β-galactosidase activity, as reported by Errington and Vogt (1990).

*Competency and transformability*. As these morphotypes and group I were drug resistant, we tested if they were able to transfer $Cm^R$ and $Erm^R$ into *B. subtilis*. However, chromosomal DNA of *C*, *T*, *V* and *SV* morphotypes and of the four group I *Bacilli* did not transform drug-sensitive *B. subtilis* recipients to drug resistance or prototrophy. Conversely, attempts to transform the *B. subtilis* $Rif^R$ and prototrophic markers into competent *C*, *T*, *V* and *SV* morphotypes failed. The lack of transformability could be the result of inhomologies between genetic loci or lack of homologous loci, as borne out by the Southern hybridizations for the *leu* and *thr* genes shown in Fig. 3B. It was not possible to select for transformants of conserved genes (i.e. integrative plasmids containing conserved *rrn* fragments marked with $Cm^R$; Jarvis *et al*., 1988), as these morphotypes and group I *Bacilli* are already drug resistant.

*Sporulation and spore morphology*. The *C*, *T* and *V* morphotypes sporulated in liquid nutrient sporulation medium (NSM; Schaeffer *et al*., 1965) at similar frequencies to *B. subtilis* (data not shown). Electron microscopy of spore thin sections revealed differences in spore morphology. The dense outer coat of a *B. subtilis* spore is uniformly constant (Fig. 5A, upper left corner). However, the *C*, *T* and *V* types (Fig. 5B, C and D respectively) have an additional spongy coat and were composed of five spikes distributed either equally for *C* or asymmetrically for *T* (Fig. 5B and C respectively) as seen in group I *Bacilli*, such as *B. popillae* and *B. cereus* (Fig. 5E, bottom; Aronson and Fitz-James, 1976). The *V* spore had four symmetrical spikes (Fig. 5D).

*Pattern formation*. The pattern-forming ability of group I *Bacilli* was examined on the two nutrient substrates we

**Fig. 6.** A. Patterns of group I *Bacilli* species after 48–72 h of growth on nutrient-poor hard agar.
Aa. Control *V* morphotype grown on 2 g $l^{-1}$ peptone, 2.5% agar incubated at 30°C. All other species were incubated at 37°C.
Ab and Ac. *B. circulans* ATCC 4513 grown on 2 g $l^{-1}$ peptone and two agar concentrations of 2.5% and 1.5% respectively. As a result, they differed in humidity.
Ad. *B. macerans* BKM B-51 grown on 2 g $l^{-1}$ peptone, 1% agar.
Ae and Af. *B. alvei* PB1875 grown on 2 g $l^{-1}$ peptone, 1.5% agar.
B. A comparison of the patterns formed by group II *Bacilli* grown on nutrient-rich soft agar–Xgal (Ba–Bc) and nutrient-poor hard agar (Bd–Bf) The former plates were TB–0.6% agar containing 160 μg $ml^{-1}$ Xgal; the latter plates were composed of 2 g $l^{-1}$ peptone, 1.5% agar.
Ba and Bd. *B. subtilis* W23 in which one *C*-like morphotype emerged after 9 days of incubation at 37°C on the surface of the hard plate (Bd).
Bb and Be. *B. pumilus* BD2002 and 8A1 respectively.
Bc and Bf. *B. licheniformis* FD01.
All nutrient-poor hard agar plates (Ba–Bc) were stained with brilliant blue. The nutrient-rich soft agar (TB–Xgal) plates (Bd–Bf) were not stained.


A
a
b
c
d
e
f
B
a
b
c
d
e
f

used in our initial experiments, low-nutrient high agar and high-nutrient low agar. Most members of group I *Bacilli* tested underwent pattern growth on nutrient-poor hard agar within 48 h (some are shown in Fig. 6Aa–Af). *B. circulans* (Fig. 6Ab and Ac) was similar to the *V* morphotype (Fig. 6Aa), as it had leading droplets of cells that spun in a circular motion (i.e. like a vortex) with branches migrating away from the colony centre. *B. macerans* strain BKM B-5 was similar to a highly branched *V* type (Fig. 6Ad). *B. alvei* (Fig. 6Ae and Af) exhibited a dual mode of expansion, combining features of the *C* and *V* morphotypes. Its branches migrated away from and towards the colony centre via leading vortical (*V*) droplets that chiralled (*C*) in all directions with no particular handedness (Fig. 6Ae and Af). We, therefore, designated the *B. alvei* pattern as a rotating and wandering (*RW*) morphotype. A similar description of *B. alvei* growth on hard agar surfaces has been described by Henrichsen (1972).

We also examined the pattern-forming ability of the following group II *Bacilli*: *B. globigii*, *B. pumilus*, *B. licheniformis*, *B. amyloliquifaciens*, W23, a *B. subtilis* relative, and wild-type *B. subtilis* ATTC 3610. Like *B. subtilis*, all generated elaborate patterns on nutrient-rich soft agar plates (some are shown in Fig. 6Ba–Bc). W23 generated a layered radial (*LR*) pattern on nutrient-rich soft agar plates (Fig. 6Ba) similar to *B. subtilis* strain BD79 described above (Fig. 2C); *B. pumilus* generated a highly branched *V*-like pattern (Fig. 6Bb); wild-type *B. subtilis* NCTC3610 exhibited a non-defined swarming morphology (not shown). These strains did not generate patterns on the nutrient-poor hard agar plates (W23 in Fig. 6Bd and *B. pumilus* in Fig. 6Be) beyond the minimum branching seen for the *B* morphotype of *B. subtilis* (Fig. 1A). One exception is *B. licheniformis*, which gave a feathery pattern on nutrient-poor hard agar plates (Fig. 6Bf) and some weak β-galactosidase activity on plates containing Xgal (Fig. 6Bc). As found in our initial *B. subtilis* inoculations, a few late-arising *T* morphotypes emerged from aged W23 colonies (1/9; Fig. 6Bd).

Because the 16S DNA homology between *C*/*T* and *B. alvei* or *V* and *B. macerans* was high (95%), we assumed that they would form similar patterns. However, they were dissimilar, suggesting that the *C*, *T*, *V* and *SV* morphotypes were not *B. alvei* or *B. macerans*. To prove it, we performed comparative Southern hybridizations and resequenced a few 16S *rrn*s of some group I *Bacilli*.

*Southern hybridizations and 16S sequencing*. The *Eco*RI *rrn* restriction profiles of *B. alvei* and *B. macerans* did not resemble the *C*/*T* or *V* morphotypes (Fig. 7). These comparisons were complicated by an additional factor, namely strains from different sources showed different hybridization patterns. For example, two *B. alvei* strains (PB1875 and B-383) and two *B. macerans* strains (BKM B-51 and B-4267) gave different band patterns from each other (Fig. 7). Others, such as *B. alvei* and *B. popillae*, essentially shared total homology with each other in both their ribosomal restriction (Fig. 7) and morphotype patterns. Thus, we sequenced the 16S DNA of some of these group I *Bacilli* strains and found that *B. macerans* BKM B-51 was really *B. cereus* and *B. alvei* PB1875 did not closely match anything in the database, but still gave a *B. alvei RW*-like morphotype. All still fell within the group I *Bacilli* with more than 90% sequence identity to each other, true sequence identity with those found in the DNA database and only 82–85% sequence identity with the '*B. subtilis*' group. All our sequences from group I *Bacilli* did not show identity with *T*/*C* or *V*.

*Group I detectability*

Our results above as well as our own inspection of

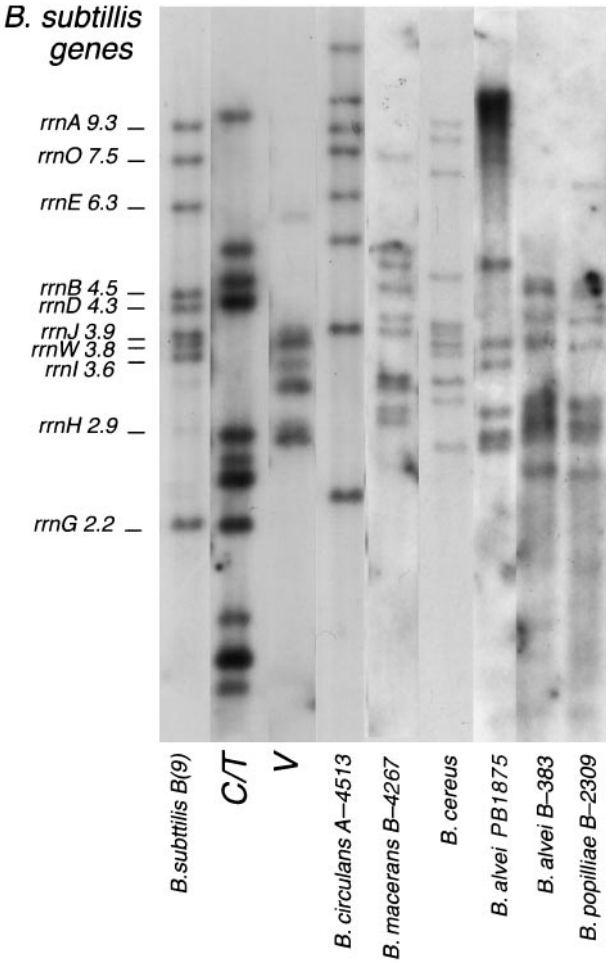



**Fig. 7.** Southern blots of *Eco*RI-digested chromosomal DNAs from group I and the *T*, *C* and *V* morphotypes hybridized with the *B. subtilis rrn* probe. The DNAs were digested with *Eco*RI, electrophoresed on 0.8% agarose and, after transfer, probed with a labelled 23S-5S fragment as described in *Experimental procedures*. Compare the hybrid bands with those shown in Fig. 3A.

**Table 2.** The critical number of cells needed to produce *T*/*C*/*V* patterns on nutrient-poor hard agar[a].

| | A. Unmixed cultures No. of cells/inoculation[b] | | | | B. Mixed cultures No. of cells/inoculation + B cells ($7.5\times10^5$)[b] | | |
|---|---|---|---|---|---|---|---|
| *T* | 750 | 75 | 7 | *B*+*T* | 650 | 65 | 33 |
| | 6/6 | 6/6 | 3/6 | | 9/9 | 9/9 | 0/9 |
| *C* | 330 | 33 | 12 | *B*+*C* | 450 | 45 | 20 |
| | 6/6 | 6/6 | 3/6 | | 9/9 | 9/9 | 0/9 |
| *V* | 8500 | 850 | 260 | *B*+*V* | 1500 | 150 | 75 |
| | 6/6 | 3/6 | 0/6 | | 9/9 | 3/9 | 0/9 |

**a.** Liquid cultures of *T*, *C* and *V* morphotypes were diluted to $10^{-4}$, $10^{-5}$ and $10^{-6}$ for both viable counts and for inoculations of 5 μl on nutrient-poor hard agar plates. An undiluted culture of strain BD170 was mixed with the diluted morphotypes and inoculated similarly (B).
**b.** Upper rows represent total number of plated cells in each dilution (i.e. 750 to 7 for *T* unmixed). Lower rows represent the numbers of point-inoculated colonies that produced the morphotype pattern (i.e. 6/6 and 3/6 yielded a *T* pattern when 75 and 7 cells were inoculated respectively; or 6/6 and 3/6 yielded a *C* pattern when 33 and 12 cells were inoculated respectively).

previously published reports (Fujikawa and Matsushita, 1989; Ben Jacob *et al*., 1992; 1994a; 1995a; Fujikawa, 1994) suggested to us that members of group I *Bacilli* can co-exist with group II *Bacilli* and go unnoticed. To determine if this was possible, we purposely mixed the two groups and examined pattern emergence. Group I morphotypes (*T*, *C* and *V*) were diluted to numbers ranging from 10 to 1500 cells, mixed with a large population of *B. subtilis* cells ($7.5\times10^5$) of BD170 and point inoculated on nutrient-poor hard agar plates. Unmixed (*T*, *C* and *V*) control populations showed a low but critical number of cells necessary to initiate patterning reliably in a 48–72 h period: for *C*, it was approximately 33 cells, for *T* approximately 75 and, for *V*, more than 1000 (Table 2A). The mixed populations revealed the patterns faster: in 24 h with no difference in the critical number of cells required for *C* and *T* types but a lower requirement for the *V* type (compare 150 with 850 in Table 2A and B).

Table 3 lists all *Bacillus* species used in this study and summarizes our search for group I morphotypes among the various *B. subtilis* strains from different sources. All were tested on nutrient-poor hard agar with multiple point inoculations (at least nine spots on at least 12 parallel plates) and examined after long incubation periods, as described above. As shown, one to three group I morphotypes emerged from at least half of the *B. subtilis* strains (Table 3A). We did not see a correlation between key genetic backgrounds or strain sources. We observed, as did Ben-Jacob *et al*. (1997), subtle differences in the 'design' of *T* patterns generated from different *B. subtilis* strains. As shown in Fig. 8, on the same nutrient-poor hard agar plate, *T* morphotypes isolated from strains 168T, BD170 and IS56 gave rise to a variety of patterns all exhibiting tip-splitting growth. DNAs prepared from these morphotypes gave identical and reproducible *T* Southern hybridizations with the conserved 23S-5S probe (see Fig. 3A), showing that, even among a group I morphotype species, there are strain variations in the pattern produced.

## Discussion

This paper clarifies potentially confusing issues for those who study, or wish to study, in *Bacilli*, one of the most naturally common forms of bacterial growth, pattern formation on solid and semi-solid surfaces. We have demonstrated that a number of studies, which claimed to characterize pattern growth in *B. subtilis* (reviewed in Matsuyama and Matsushita, 1993; Ben-Jacob, 1997; Ben-Jacob and Cohen, 1997), were probably not working with *B. subtilis* under certain conditions, but with a different class of *Bacilli* altogether, called group I. We have also shown how it is

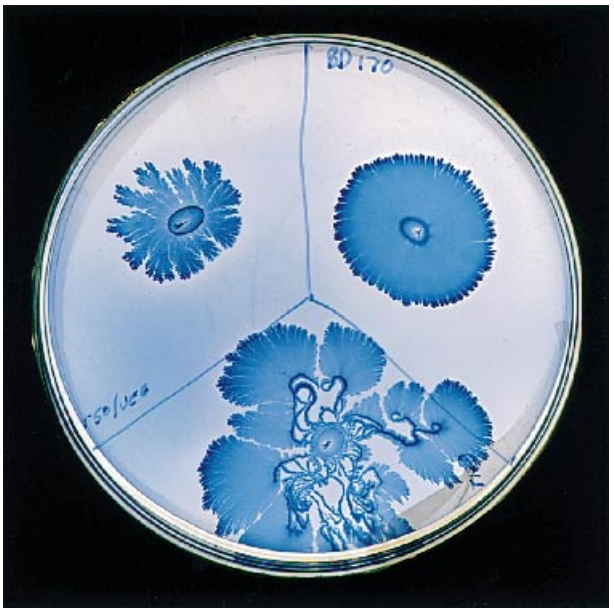



**Fig. 8.** Comparison between patterns developed by three group I *T* morphotypes that emerged from different *B. subtilis* strains. The growth conditions are 1.25 g $l^{-1}$ peptone, 1.25% agar incubated at 37°C for 48 h. Beginning with the upper left colony, original *B. subtilis* strains were IS58/pPW810-*veg*, BD170/pBSO2a-*thr* and 168T respectively.

**Table 3.** *Bacillus* species used in the study of pattern formation on hard agar.

| *Bacillus* strain[a] | Genotype[b] | Emergent patterns[c] | Origin[d] |
|---|---|---|---|
| A. Group *II,* B. subtilis | | | |
| 168T | *trpC2* | *T*,*C*,*V*[c] | K. Bott |
| NCTC3610 | Prototroph | None | A. Sonenshein |
| W23 | Prototroph, *Sm*$^r$, *Er*$^r$,*Mic*$^r$ | *T* | D. Dubnau |
| BD170 | *trpC2*, *thrA5-/+ pBSO2a-thr* | None, *T* | This work (D. Dubnau) |
| BD79 | *leuB1*, *pheA1-/+ pER102-leu* | *T*, *C* | This work (D. Dubnau) |
| SB25 | *trpC 2*, *hisH2* | *T*, *C*, *V*[c] | D. Dubnau |
| IS58 | *trpC2*, *lys-3 −/+ pPW810-veg* | *T*, *C* | This work (I. Smith) |
| IS56 | *trpC2*, *lys-3*, *relA −/+ pPW810-veg* | *T*, *C*, *V*[c], *SV*[c] | This work (I. Smith) |
| YS1 | *purB6*, *leuA8*, $R_m^+$, $M_m^+$ | *T* | D. Dubnau |
| RM125 | *arg15*, *leuA8*, $R_m^-$,$M_m^-$ | *T* | K. Nagahari |
| 1A718 | *trpC2*, *his*, *met*, *try-1*, *ura*, *Km*, *lacZ-ΔM15*, *rib* $R_m$, $M_m^+$ | None | BGSC (S. Bron) |
| 1A685 | *trpC2*, *met*, *his*, *tyr-1*, *ura*, *rib*,$R_m$,$M_m^+$ | None | BGSC (P. Haima) |
| 1A698 | *pheA1*, *sfp* | None | BGSC (P. Zuber) |
| 1A699 | *pheA1*, *sfp*, *srfA::Tn917* | None | BGSC (P. Zuber) |
| BD1890 | *his*, *leu*, *met*, *srfA::Tn917* | None | D. Dubnau |
| OI1085 | *trpC2*, *met*, *his*, *ch*$^{e+}$ | *T* | G. Ordal |
| OI2836 | *trpC2*, *met*, *cheB* | *T* | G. Ordal |
| OI1055 | *trpC2*, *met*, *cheY* | *T* | G. Ordal |
| OI3180 | *trpC2*,*Δ four receptors* | *T* | G. Ordal |
| BD224 | *trpC2*, *thrA5*, *recA4* | None | D. Dubnau |
| JH646 | *trpC2*, *pheA1*, *spoOA12* | None | I. Smith |
| *B. Group II, other* | | | |
| *B. globigii* | | | |
| RUB562 | Prototroph | None | C. Tackney |
| *B. pumilus* | | | |
| 8A1 | Prototroph, *hsrP*$^+$ | None | BGSC (F. Young) |
| BD2002 | Prototroph | None | D. Dubnau |
| Bp | *trpE* | None | D. Henner |
| *B. licheniformis* | | | |
| FDO1 | Prototroph, *Sm*$^r$ | *T* | D. Dubnau |
| ACTC848 | Prototroph, *Sm*$^r$ | None | A. Garro |
| *B. amyloliquefaciens* | | | |
| H | Prototroph | *T* | A. Garro |
| *C. Group I*, *other* | | *Natural patterns* | |
| *B. circulans* | | | |
| ATCC 4513 | Prototroph | *V*-like | BGSC |
| NRRL B-378 | Uncharacterized | *V*-like | L. Nakamura |
| *B. macerans* | | | |
| BKM B-51 | Prototroph | *T*-*C*-like | BGSC |
| NRRL B-4267 | Uncharacterized | None | L. Nakamura |
| *B. alvei* | | | |
| PB1875 | Uncharacterized | *RW* | A. Galizzi |
| NRRL B-383 | Uncharacterized | *RW* | L. Nakamura |
| *B. popillae* | | | |
| NRRL B-2309 | Uncharacterized | *RW* | L. Nakamura |

**a.** Group I and II *Bacilli* according to the classification of Piest (1993); the group II were previously studied by us (Gottlieb and Rudner, 1985).
**b.** Four strains contained plasmids introduced by us, and they were pBSO2a-*thr*, pER102-*leu* and pPW810-*veg*, a derivative of pDH32 (see *Experimental procedures*).
**c.** The original *T*, *C*, *V* and *SV* were generated in Ben-Jacob's laboratory using strain 168T, and we provided them with strains IS58 and IS56 with and without pPW810-*veg*. The *V* and *SV* were from their collection. *RW*, rotating and wandering pattern morphology (see Fig. 6Ae–Af).
**d.** BGSC, Bacillus Genetic Stock Center, Columbus, OH, USA; the names in parentheses represent the individuals who isolated some of these strains. NRRL, National Center for Agricultural Utilization Research, Peoria, IL, USA.

possible, even simple, for these other species to go undetected when co-cultured at low frequencies with *B. subtilis*. The 16S rDNA sequences, the Southern hybridizations and the phenotypic characterizations clearly showed that the *C*, *T* and *V* morphotypes belong to group I *Bacilli* and are probably newly described species. We, thus, name the *C*/*T* morphotype as *Bacillus tipchirales* and the *V* morphotype as *Bacillus vortex*. This conclusion leads to a revision of several mechanistic hypotheses that have been proposed to explain *B. subtilis* pattern growth. One such proposal views the bacterial genome as an adaptive cybergenetic unit with 'self interest' and is based on the finding that, once the pattern is generated, it is then propagated (Ben-Jacob *et al.*, 1994b; Ben-Jacob, 1997). According to this theory, mutations and genome rearrangements occur under hostile environmental

conditions, as instructed by 'cybernators', leading to the formation of a colonial pattern. We now know that this is not the case. A *T* pattern stays a *T* under these conditions and likewise for the *B*; there is no conversion from one to the other, because they are different *Bacillus* species.

A second proposal, which is supported by this study, concerns the role of humidity (Matsuyama and Matsushita, 1993; Mendelson and Salhi, 1996). According to this mechanism, the essential requirement for the morphogenesis of complex forms is 'restraint of cell motility', which is governed by the wetness of the local environment (Mendelson and Salhi, 1996). Unrestrained states (high wetness) allow swarming but little colony structure, whereas partially restrained states (within the wetness boundaries) foster the growth of complex patterns with fractal properties, and totally restrained states (low wetness) yield small compact colonies of circular form. Our study suggests that different groups of *Bacilli* have different restraint boundaries. Group I morphotypes have more defined patterns on harder agar surfaces (low wetness), whereas group II morphotypes have rich patterns on softer agar (high wetness). Perhaps the wetting agents, i.e. surfactin-like compounds (Matsuyama and Matsushita, 1993; Zuber *et al*., 1993), of group I *Bacilli* are better adapted to drier conditions than those of group II.

Another issue we addressed relates to the optimal conditions for the selection of group I patterns among *B. subtilis* populations. It appears that group I *Bacilli* can co-exist with *B. subtilis* and go unnoticed, or are inhibited, when grown on conditions favourable for *B. subtilis*. When grown in an unfavourable environment (low nutrient, low humidity), various group I morphotypes emerge from the *B. subtilis* inoculations. A number of previous studies, largely initiated by physicists, examined patterned growth in *B. subtilis* using these unfavourable conditions (Fujikawa and Matsushita, 1989; Ben-Jacob *et al*., 1992; 1994a,b; Fujikawa, 1994). They compared their findings with fractal-like growths seen in non-living systems, such as solidification from saturated solutions, electrochemical depositions, snowflake formation, etc. (Mandelbrot, 1983; Fujikawa and Matsushita, 1989; Viscek, 1989; Ben-Jacob and Garik, 1990). They proposed models, such as diffusion-limited aggregation (DLA; Witten and Sanders, 1981), the Eden (Peters *et al*., 1979) and the dense-branching morphology (DBM) models (Ben-Jacob *et al*., 1986) to explain the *B. subtilis* growth patterns. Apparently, these non-traditional growth conditions led to the isolation of the *T*, *C*, *V* and *SV* morphotypes. Likewise, the pattern morphologies we have shown in the current study, on similar nutrient-poor high agar plates, are nearly identical to the *C*, *T* and *V* patterns shown in the original studies (see Fig. 5 in Matsuyama and Matsushita, 1993; and Fig. 1 in Ben-Jacob *et al*., 1994b).

One of the pertinent questions resulting from our study is how the *C*, *T*, *V* and *SV* bacteria become associated with *B. subtilis* and how they are able to remain undetected. We suggest two possibilities: either we are simply dealing with chronic contamination or a natural co-existence. If an 'introduced' contaminant, it had to occur in *B. subtilis* strains more than 10 years ago when studies on *T* and similar morphotypes were under way (Fujikawa and Matsushita, 1989). That is, although our laboratory had exchanged strains with Ben-Jacob (see Table 3), neither we nor Ben-Jacob (personal communication) have done so with other investigators (Fujikawa and Matsushita, 1989; Matsuyama *et al*., 1989). However, it is difficult for us to dismiss the morphotypes as mere contaminants for the following reasons: (i) only members of group I *Bacilli* emerged from the *B. subtilis* suspensions, i.e. no other species or genus of bacteria or fungi were identified; (ii) no other growth was seen on the Petri dishes outside of the inoculated suspensions, i.e. spores in the air that might happen to fall into the plate cannot explain our findings; (iii) the appearance of these morphotypes has emerged from *B. subtilis* suspensions in different laboratories around the world; in Japan (Matsuyama *et al*., 1989), Israel (Ben-Jacob *et al*., 1992) and New York (this study); (iv) *B. subtilis* strains from a variety of sources, such as the *Bacillus* Genetic Stock Center (BGSC), the National Center for Agricultural Utilization Research (NRRL) and colleagues, showed emergence of the patterns (Table 3); and (v) a *T*-like pattern even emerged on low-nutrient low agar from a *B. subtilis* culture purified from food in Japan (see Fig. 2 in Fujikawa, 1994). What we may be observing here, but as yet unproven, are the remains of a natural co-existence. In natural environments, bacteria are not isolated cultures but exist as mixed populations. It is not inconceivable that certain *Bacilli* evolved permanent relationships with each other. However, if present, they will remained undetected, because most microbiologists do not grow their *B. subtilis* cultures in these unfavourable conditions.

Three hypotheses for a natural co-existence can be formulated: (i) a symbiotic carrier state in which presumably pure cultures and spore suspensions are actually mixtures of two or three species; (ii) a novel genetic state in which *Bacillus* cells can carry both expressed and silent genomes as proposed for certain non-complementing diploids of *B. subtilis* protoplast fusions (Hotchkiss and Gabor, 1985; Grandjean *et al*., 1996); and (iii) a minority state of freely isolated group I cells, which become opportunistic under unfavourable conditions and feed off of *B. subtilis*. In support of the first possibility, members of group I *Bacilli* are known for their symbiotic relationships with plant roots, sheep and the intestines of the common sow bug (*B. polymxa*, *B. macerans* and *B. cereus* respectively; Gordon, 1973; Piest, 1993; Jorgensen *et al*., 1997). One may test these hypotheses either by micromanipulating

single cells/spores and ascertaining their progeny pattern(s) propensity or by the use of fluorescent oligonucleotide probes against unique sequences of the companion species, i.e. the 16S rDNA variable region, and detecting its presence in a *B. subtilis* population. Ribosomal RNA-based probes have been used successfully for rapid phylogenetic identification of single microbial cells in artificial mixtures (DeLong *et al*., 1989; Amann *et al*., 1990) and in complex phylogenetic analyses of unusual microbes, such as the *Metabacterium polyspora* (Andert *et al*., 1996).

In closing, the results of this study are useful in gaining a better understanding of the current body of literature on pattern formation in *Bacilli* and are essential before one can begin to address the molecular genetics of pattern formation in this genus. Although it is not yet known what purpose pattern formation serves, the choice of conditions and, thus, the species one is bound to work with, especially when there is potential for co-existence, is paramount in designing useful experiments. The issues addressed in this study also have health-related interest. The *T*, *C*, *V* and *SV* morphotypes are intriguing not only because of their unique multicellular organization, but also because of their phenotypic multiple antibiotic resistance character. This resistance, we suggest, could be related to the secretion of the 'wetting' fluids associated with motility on hard surfaces containing antibiotics. Multiple drug resistance has been described extensively for plasmid-borne R factors and viewed as a dangerous reservoir for transfer to pathogenic organisms. However, the nature of the resistance property described here, expressed only on hard surfaces, may represent a new mechanism. Thus, these morphotypes could represent a health hazard, not only because of their high drug resistance, but also because of their potential long-range ability to survive, co-exist with and be mistaken for drug-sensitive *Bacilli* species.

## Experimental procedures

### *Media and growth conditions for pattern formation*

All strains were maintained as streaks on plates composed of 33 g of tryptose blood agar base (TBAB; Difco) plus an additional 5 g of bacto agar (Difco), 0.05 M $MgSO_4$ and 50 μM MnCl $l^{-1}$ deionized water. TBAB consists of 10 g tryptose, 3 g beef extract, 5 g NaCl and 15 g agar per 33 g. For routine growth in liquid cultures, either VY, composed of 2.5% veal infusion (Difco) and 0.5% yeast extract (Difco), or Luria broth (LB) was used. Media used to produce patterns were as follows.

*Nutrient-poor hard agar plates*. These were prepared and designated as described by Ben-Jacob *et al*. (1992). Specifically, the ones used in the study were prepared by mixing 5 g $l^{-1}$ NaCl, 5 g $l^{-1}$ $KHPO_4$, 1–2.5 g $l^{-1}$ bacto peptone (Difco) and 10–25 g $l^{-1}$ bacto agar (Difco) in deionized water. For optimal pattern formation, the agar concentration and temperature conditions for *C* morphotypes is 1.25% agar incubated at 37°C, for *T*, 1.5–1.75% at 37°C and, for *V*, 2–2.5% at 30°C. The *V* morphotypes appear faster with 15 g $l^{-1}$ bacto peptone (Ben-Jacob *et al*., 1997).

*Nutrient-rich soft agar plates*. These were prepared according to Mendelson and Salhi (1996) and were similar to TBAB plates but with low agar. The ones we used were composed of 10 g $l^{-1}$ tryptose (Difco), 3 g $l^{-1}$ beef extract (TB) or veal infusion broth (TV), 6 g $l^{-1}$ bacto agar and 5 g $l^{-1}$ NaCl in deionized water. Before pouring, 160 μg $ml^{-1}$ Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), diluted from a 160 mg $ml^{-1}$ stock solution in *N*,*N*-dimethylformamide (Fisher Scientific), was added. When strains harbouring integrated plasmids were used, either chloramphenicol at 10 μg $ml^{-1}$ or erythromycin and lincomycin (Sigma) at 1 and 25 μg $ml^{-1}$ were added. Both types of pattern plates were poured when the medium was cooled to and held at 48°C for at least 1 h. For nutrient-poor hard agar, 12.5 or 15 ml was than dispensed into 100-mm-diameter plastic Petri dishes. For the nutrient-rich soft agar, 25 ml was dispensed. Before inoculation, plates were dried for 2–3 days at room temperature until they lost 5% of their initial weight. For the nutrient-rich soft agar, inoculations before this time led to swarming for some strains and after this time to a more condensed, sometimes compact, growth as detailed in Mendelson and Salhi (1996).

To generate patterns, 'single' bacterial colonies were picked from a streak on a TBAB plate grown overnight in 2.0 ml of LB or VY broth at 37°C (30°C for *V* bacteria), followed by two washes with dilution salts (0.15 M NaCl, 50 mM $NaPO_4$, pH 7.4) and resuspended in 2 ml of dilution salts (approximately $5 \times 10^8$ cells $ml^{-1}$). The starting cell titres of *B. subtilis* and *T*, *C* and *V* morphotypes used to generate patterns or in mixing experiments were confirmed by viable platings on LB or nutrient agar. The plates were inoculated with either one, two, four or nine droplets (5 μl) each containing $1–5 \times 10^5$ washed bacteria and incubated at 37°C (30°C for *V* bacteria) with 30–40% humidity for periods ranging from 2 to 21 days. Growth was monitored daily and also visualized microscopically using either an Olympus optical microscope (up to ×500 magnification) or a Nikon Fluorphot microscope (up to ×600 magnification). To prevent further growth and to analyse pattern morphology, those on nutrient-poor hard agar plates were stained with 0.1% brilliant blue in 50% methanol, 10% acetic acid for 60–90 s, followed by destaining with 30% methanol, 10% acetic acid for 10–20 min. This was done because it is difficult to discern pattern morphologies on the nutrient-poor thin hard agar plates, as they appear very transparent. It was not necessary to stain the nutrient-rich soft agar plates, as they contained Xgal, and the patterns are clearly visualized.

To prevent any contamination of the cultures, all the glassware was routinely washed with chromic-sulphuric acid cleaning solution to remove spores before autoclaving. Disposable plates and pipettes were used in a spore-free facility. In all experiments, media tubes that were not inoculated with cells were incubated in parallel with the culture tubes in which nothing grew, as expected when following standard microbiological techniques.

### *Phenotypic characterizations and genetic analysis*

The initial cell suspensions as well as suspensions from

bacteria toothpicked from tip branches after patterns were formed were used to characterize phenotypes. Minimal medium (MM) plates (Spizizen, 1958) were supplemented with the required growth factors for each individual strain (Table 3). When appropriate, 40 $\mu$g ml$^{-1}$ Xgal was added to the plates. Drug resistance was checked on LB plates containing either 10 $\mu$g ml$^{-1}$ chloramphenicol (Cm) or 25 $\mu$g ml$^{-1}$ lincomycin and 1 $\mu$g ml$^{-1}$ erythromycin (Erm) or 10 $\mu$g ml$^{-1}$ kanamycin (Km) or 100 $\mu$g ml$^{-1}$ ampicillin or 20 $\mu$g ml$^{-1}$ tetracycline (Tc) or 40 $\mu$g ml$^{-1}$ rifampin (Rif) or 125 $\mu$g ml$^{-1}$ spectinomycin (Sp) or 1000–2000 $\mu$g ml$^{-1}$ streptomycin (Sm). The *relA* mutant (IS56; see Table 3) was checked for sensitivity to 15–30 mM 3-amino-1,2,4-triazole (AT), a histidine analogue associated with high cellular levels of ppGpp (Gropp *et al*., 1994), according to Rudd *et al*. (1985). New biochemical requirements were identified by auxanography using nine mixtures, each containing four types of growth factors (amino acids, purines, pyrimidines and vitamins; Holliday, 1956). The vitamin mixture contained (in $\mu$g ml$^{-1}$) thiamine HCl, 300; nicotinic acid, 750; riboflavin, 150; p-amino benzoic acid, 150; pyridoxine HCl, 300; and biotin, 50. Enriched minimal medium (enMM), used for comparative growth rates, contained MM medium supplemented with 0.5% glucose, 1% sodium glutamate, 0.05% yeast extract (Difco), 0.02% vitamin-free casamino acids (Difco) and the required amino acids of the original *B. subtilis* strains. Bacterial growth in liquid cultures was followed on a Klett–Summerson spectrophotometer equipped with a red filter at readings in the range of 20 to 250. Viable counts for group I detectability assays (Table 2) were performed by plating the appropriately diluted cultures on LB or nutrient broth (NB) plates.

Sporulation frequency was determined after overnight growth at 37°C in nutrient sporulation medium (NSM), as described by Schaeffer *et al*. (1965). The percentage of viable cells was assayed after heating serially diluted cells in 1 ml samples for 10–20 min at 80°C and plating onto LB plates. Electron microscopy of spores, purified by lysozyme-SDS treatments (Margulies *et al*., 1978), was performed following the procedures of Barak and Youngman (1996). Embedded samples were sectioned at 40–120 nm thickness on a MT-2 ultramicrotome (LKB). Sections were stained with uranyl acetate drops for 20 min followed by lead citrate drops for 6 min. Stained sections were examined and photographed on a H-600 electron microscope. To check the development of competency in the pattern-producing strains and the ability of their DNA to transform *B. subtilis*, freshly prepared competent cells were prepared according to the two-step shift down procedure, as described by Anagnostopoulos and Spizizen (1961) and Rudner *et al*. (1967).

### *DNA manipulation*

DNA preparations were carried out as described previously (LaFauci *et al*., 1986). Plasmid DNA was purified from *Escherichia coli* cultures essentially according to the procedure of Tanaka and Weisblum (1975). Chromosomal DNA from *B*, *T*, *C* and *V* strains were isolated from purified cells picked from the pattern-forming plates and grown overnight in VY medium (500 ml). The *T*, *C* and *V* strains were resistant to lysozyme (2 mg ml$^{-1}$). Thus, lysis was accomplished in 2.5% SDS with heating for 10 min at 60°C, followed by purification according to standard procedures (Sambrook *et al*., 1989). Chromosomal DNA recoveries from the *T*, *C* and *V* strains were consistently two- to threefold lower than that from *B. subtilis*. For Southern hybridizations, chromosomal DNA (5–10 $\mu$g ml$^{-1}$) was digested with *Eco*RI and processed as described by Rudner *et al*. (1994) using as probes inserts from the following three plasmids: (i) pER102-*leu* containing a 3.2 kb insert of the *leuBAC* operon (LaFauci *et al*., 1986); (ii) pBSO2a-*thr* containing a 1.5 kb insert of the *thrA* gene (Rapaport *et al*., 1978); and (iii) pYR104-*rrn* containing a 2.3 kb insert of 23S-5S rDNA (Jarvis *et al*., 1988). Plasmid pPW810-*veg*, a derivative of pDH32 (Shimotsue and Henner, 1986), contains the *veg* promoter (Ollington and Losick, 1981) fused to a *lacZ* gene and was kindly given to us by C. Stewart of Rice University. This plasmid was used to mark strains with drug resistance and a *lacZ* fusion, integrated at the *amyE* locus. The DNA fragments for the probes and those produced by PCR were isolated from gels using the Qiaex Gel Extraction Kit (Qiagen). Probes were labelled with the DECAprime II DNA Labeling Kit as directed by the supplier (Ambion) using either [$\alpha$-$^{32}$P]-dCTP or dATP.

### *16S ribosomal RNA gene amplification and sequencing*

Amplification of 16S rDNA was performed according to Suzuki and Yamasato (1994) with a Perkin-Elmer Cetus thermocycler in 100 $\mu$l reactions containing 100 ng of chromosomal DNA, 1× PCR buffer I (Perkin-Elmer Cetus), 200 $\mu$M each dNTP (Pharmacia LKB), 1 $\mu$M each primer and 5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) or Vent DNA polymerase (New England Biolabs). The primers were: 5′-GAGTTTGATCCTGGCTCAG (*E. coli* numbering positions 9–27) and 5′-AGAAAGGAGGTGATCCAGCC (positions 1525–1544). PCR amplification included an initial denaturation step at 94°C for 2.5 min, followed by 30 cycles of 94°C for 1 min, 58°C for 2.5 min, then 72°C for 2.5 min and a final extension step at 72°C for 5 min. The amplified products were purified by gel electrophoresis on 1% agarose and recovered from the gels as mentioned above. In addition to those above, internal primers constructed for further sequencing were: 5′-CGTGCCAGCAGCCGCGGT (positions 529–546) and 5′-CACTGACGGCCACTGT (positions 881–897). DNA sequencing was performed using [$\alpha$-$^{35}$S]-dATP (Dupont-NEN) and the Thermocycler sequencing kit (Perkin-Elmer). The sequences were aligned to the *B. subtilis* 16S *rrnA* gene reported in GenBank. Percent homology calculations did not include ambiguous nucleotides (N).

## Acknowledgements

This paper is dedicated to Dean Erwin Fleissner to honour his retirement from Hunter College and to celebrate his many contributions to the Division of Sciences and Mathematics. We thank Ludmila Haimovich for assistance with the electron microscopy studies, Victoria Richmond-Chew, Minha Wang, Linda Adepoju and D.M.L. for assistance, for critically evaluating the results and for moral support. We thank E. Ben-Jacob for providing us with strains and useful discussions during the initial stages of this project. This work was first presented, in part, at the Wind River Conference on Prokaryotic

Biology, June, 1996. This investigation was supported by a Research Centers in Minority Institutions award RR-03037 from the National Center for Research Resources, NIH, and by City University of New York Faculty Research awards 665148 and 667198.

## References

Amann, R.I., Krumholz, L., and Stahl, D.A. (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol* **172:** 762–770.

Anagnostopoulos, C., and Spizizen, J. (1961) Requirements for transformation in *Bacillus subtilis*. *J Bacteriol* **81:** 741–745.

Angert, E.R., Brooks, A.E., and Pace, N.R. (1996) Phylogenetic analysis of Metabacterium polyspora: clues to the evolutionary origin of daughter cell production in Epuloscium species, the largest bacteria. *J Bacteriol* **178:** 1451–1456.

Aronson, A.I., and Fitz-James, P. (1976) Structure and morphogenesis of bacterial spore coat. *Bacteriol Rev* **40:** 360–402.

Ash, C., Farrow, J.A.E., and Collins, M.D. (1991) Phylogenetic heterogeneity of the genus Bacillus revealed comparative analysis of small subunit ribosomal RNA. *Lett Appl Microbiol* **13:** 202–206.

Barak, I., and Youngman, P. (1996) SpoIIE mutants of *Bacillus subtilis* comprise two distinct phenotypic classes consistent with a functional role for the SpoIIE protein. *J Bacteriol* **178:** 4984–4989.

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., and Cohen, I. (1997) Cooperative formation of bacterial patterns. In *Bacteria as Multicellular Organisms*. Shapiro, J.A., and Dwarkin, M. (eds). New York: Oxford University Press, pp. 394–416.

Ben-Jacob, E., and Garik, P. (1990) The formation of patterns in non-equilibrium growth. *Nature* **343:** 523–530.

Ben-Jacob, E., Deucher, G., Garik, P., Goldenfeld, N., and Lareah, Y. (1986) Formation of a dense branching morphology in interfacial growth. *Phys Rev Lett* **57:** 1903–1906.

Ben-Jacob, E., Shmueli, H., Schochet, O., and Tennenbaum, A. (1992) Adaptive self-organization during growth of bacterial colonies. *Phyisca A* **187:** 378–424.

Ben-Jacob, E., Schochet, O., Tennenbaum, A., Cohen, I., Czirok, A., and Vicsek, T. (1994a)Generic modelling of cooperative growth patterns in bacterial colonies. *Nature* **368:** 46–49.

Ben-Jacob, E., Tennenbaum, A., Schochet, O., and Avidan, O. (1994b) Holotransformation of bacterial colonies and genome cybernetics. *Phyisca A* **202:** 1–47.

Ben-Jacob, E., Schochet, O., Tennenbaum, A., Cohen, I., Czirok, A., and Vicsek, T. (1994c) Communication, regulation and control during complex patterning of bacterial colonies. *Fractals* **2:** 15–44.

Ben-Jacob, E., Schochet, O., Cohen, I., Tennenbaum, A., Czirok., A., and Vicsek, T. (1995a) Cooperative strategies in formation of complex bacterial patterns. *Fractals* **3:** 849–868.

Ben-Jacob, E., Cohen, I. Schochet, O., and Tennenbaum, A. (1995b) Cooperative formation of chiral patterns during growth of bacterial colonies. *Phys Rev Lett* **75:** 2899–2902.

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

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., *et al*. (1987) Bacterial biofilms in nature and disease. *Annu Rev Microbiol* **41:** 435–464.

Delong, F.E., Wickham, G.S., and Pace, N.R. (1989) Phylogenetic strains: ribosomal RNA-based probes for the identification of single cells. *Science* **243:** 1360–1363.

Errington, J., and Vogt, C.H. (1990) Isolation and characterization of mutations in the gene encoding an endogenous *Bacillus subtilis* β-galactosidase and its regulator. *J Bacteriol* **172:** 488–490.

Ford, W.W. (1916) Studies on aerobic spore-bearing non-pathogenic bacteria. Part ii: miscellaneous cultures. *J Bacteriol* **1:** 518–526.

Fujikawa, H. (1994) Diversity of the growth patterns of *Bacillus subtilis* colonies on agar plates. *FEMS Microbiol Ecol* **13:** 159–168.

Fujikawa, H., and Matsushita, M. (1989) Fractal growth of *Bacillus subtilis* on agar plates. *J Phys Soc Jpn* **58:** 3875–3878.

Gause, F.G. (1939) Some physiological properties of dextral and of sinistral forms in *Bacillus mycoides* flugge. *Biol Bull Woods Hole, MA* **76:** 448–465.

Gordon, R.E. (1973) *The Genus Bacillus*. Washington, DC: United States Department of Agriculture.

Gottlieb, P. (1984) Conserved genetic regions within the *Bacillus* chromosome. PhD dissertation, City University of New York, New York.

Gottlieb, P., and Rudner, R. (1985) Restriction site polymorphism of ribosomal ribonucleic acid gene sets in the genus *Bacillus*. *Int J Syst Bacteriol* **35:** 244–252.

Grandjean, V., LeDerout, J., and Hirschbein, L. (1996)-Non-complementing diploids from *Bacillus subtilis* protoplast fusion: relationship between maintenance of chromosomal inactivation and segregation capacity. *Genetics* **144:** 871–881.

Gropp, M., Eizenman, E., Glaser, G., Samarrai, W., and Rudner, R. (1994) A *relA*$^{(S)}$ suppressor mutant allele of *Bacillus subtilis* which maps to *relA* and responds only to carbon limitation. *Gene* **140:** 91–96.

Henrichsen, J. (1972) Bacterial surface translocation: a survey and a classification. *Bacteriol Rev* **36:** 478–503.

Holliday, R. (1956) A new method for the identification of biochemical mutants of micro-organisms. *Nature* **178:** 987–989.

Hotchkiss, R.D., and Gabor, M.H. (1985) Protoplast fusions in *Bacillus* and its consequences. In *The Molecular Biology of the Bacilli*. Dubnau, D.A. (ed.). Orlando: Academic Press, pp. 109–149.

Jarvis, E.D., Widom, R.L., LaFauci, G., Setoguchi, Y., Richter, I.R., and Rudner, R. (1988) Chromosomal organization of rRNA operons in *Bacillus subtilis*. *Genetics* **120:** 625–635.

Jorgensen, J., Dolan, S., Haselton, A., and Kochinsky, R.

(1997) Isolation and cultivation of spore- forming filamentous bacteria from *Pocellio scaber*. *Can J Microbiol* **43:** 129–135.

LaFauci, G., Widom, R.L., Eisner, R.L., Jarvis, E.D., and Rudner, R. (1986) Mapping of ribosomal RNA genes with integrable plasmids in *Bacillus subtilis*. *J Bacteriol* **165:** 204–214.

Mandelbrot, B.B. (1983) *The Fractal Geometry of Nature*. New York: W.H. Freeman & Co.

Margulies, L., Setoguchi, Y., and Rudner, R. (1978) Asymmetric transcription during post- germinative development of *Bacillus subtilis* spores. I. Hybridization patterns. *Biochim Biophys Acta* **521:** 708–718.

Matsuyama, T., and Matsushita, M. (1993) Fractal morphogenesis by a bacterial cell population. *Crit Rev Microbiol* **19:** 117–135.

Matsuyama, T., Masakazu, S., and Nakagawa, Y. (1989) Fractal spreading growth of *Serratia marcesans* which produces surface active exolipids. *FEMS Microbiol Lett* **16:** 243–246.

Mendelson, N.H., and Salhi, B.J. (1996) Patterns of reporter gene expression in phase diagram of *Bacillus subtilis* colony forms. *Bacteriology* **178**: 1980–89.

Ogasawara, N., Sieki, M., and Yoshikawa, H. (1983) The structure and organization of rRNA operons at the region of the replication origin of the *B. subtilis* chromosome. *Nucleic Acids Res* **11:** 6301–6318.

Ollington, J.F., and Losick, R. (1981) A cloned gene that is turned on at an intermediate stage of spore formation in *Bacillus subtilis*. *J Bacteriol* **147:** 443–451.

Peters, H.P., Stauffer, D., Holters, H.P., and Loewenich, K. (1979) Radius, perimeter, and density profile for percolation clusters and lattice animals. *Z Phys Rev B34* **58:** 399–408.

Piest, F. G. (1993) Systematics and ecology of *Bacillus*. In Bacillus subtilis *and Other Gram-positive Bacteria: Physiology, Biochemistry, and Molecular Genetics*. Sonenshein, A.L., Hoch, T.A., and Losick, R. (eds). Washington, DC: American Society for Microbiology Press, pp. 3–16.

Rapoport, G., Klier, A., Billault., A., Fargette, F., and Dedonder, R. (1978) Construction of a colony bank of *E. coli* containing hybrid plasmids representative of *Bacillus subtilis* 168 genome. *Mol Gen Microbiol* **176:** 239–245.

Rudd, K., Bechner, B.R., Cashel, M., and Roth, J. (1985) Mutation in the *spoT* gene of *S. typhimurium*: effects on *his* operon expression. *J Bacteriol* **163:** 534–542.

Rudner, R., Lin, H., Hoffman, S., and Chargaff, E. (1967) Studies on the loss and restoration of transforming activity of the DNA of *Bacillus subtilis*. *Biochim Biophys Acta* **144:** 269–275.

Rudner, R., Chevrestt, A., Bucholz, S., Studamire, B., White, A.-M., and Jarvis, E.D. (1993) Two tRNA gene associated with rRNA operons *rrnD* and *rrnE* in *Bacillus subtilis*. *J Bacteriol* **175:** 503–509.

Rudner, R., Studamire, B., and Jarvis, E.D. (1994) Determination of restriction fragment length polymorphism in bacteria using ribosomal RNA genes. *Methods Enzymol* **235:** 184–196.

Salhi, B., and Mendelson, N.H. (1993) Patterns of gene expression in *Bacillus subtilis* colonies. *J Bacteriol* **175:** 5000–5008.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schaeffer, P., Millet, J.P., and Aubert, J.-P. (1965) Catabolic repression of bacterial sporulation. *Proc Natl Acad Sci USA* **54:** 704–711.

Shapiro, J.A. (1995) The significances of bacterial colony patterns. *BioEssays* **17:** 597–607.

Shimotsue, H., and Henner, D. (1986) Construction of a single copy integration vector and its use in analysis of the *trp* operon of *Bacillus subtilis*. *Gene* **43:** 85–94.

Smith, R.N., and Clark, F.E. (1938) Motile colonies of *Bacillus alvei* and other bacteria. *J Bacteriol* **35:** 59–60.

Spizizen, J. (1958) Transformation of biochemically deficient strain of *B. subtilis* by deoxyribonucleate. *Proc Natl Acad Sci USA* **71:** 4787–4791.

Suzuki, T., and Yamasato, K. (1994) Phylogeny of spore-forming lactic acid bacteria based on 16S RNA gene sequences. *FEMS Micrbiol Lett* **110:** 13–18.

Tanaka, T., and Weisblum, B. (1975) Construction of a colicin E1-R factor composite plasmid *in vitro*: means for amplification of deoxyribonucleic acid. *J Bacteriol* **121:** 354–362.

Viscek, T. (1989) *Fractal Growth Phenomena*. Singapore: World Scientific Publishing.

Widom, R.L., Jarvis, E.D., LaFauchi., G., and Rudner, R. (1988) Instability of rDNA operons in *Bacillus subtilis*. *J Bacteriol* **170:** 605–610.

Winogradsky, G. (1949) *Microbiologie du Sol: Problèmes et Methodes*. Paris: Masson.

Winpenny, J.W.T. (1992) Microbial systems: patterns in time and space. *Adv Microb Ecol* **12:** 469–522.

Witten, T.A., and Sanders, L.M. (1981) Diffusion-limited aggregation, a kinetic critical phenomenon. *Phys Rev Lett* **47:** 1400–1403.

Zuber, P., Nakano, M.M., and Marahiel, M. (1993) Peptide antibiotics. In Bacillus subtilis *and Other Gram-positive Bacteria: Physiology, Biochemistry, and Molecular Genetics*. Sonenshein, A.L., Hoch, T.A., and Losick, R. (eds). Washington, DC: American Society for Microbiology Press, pp. 897–916.