Molecular Microbiology (1999) **31**(4), 1275–1281

MicroCorrespondence

LytB, a novel pneumococcal murein hydrolase essential for cell separation

Sir,

Streptococcus pneumoniae is an important human pathogen that has an absolute nutritional requirement for choline. Replacement of this amino alcohol in a synthetic medium by structural analogues, such as ethanolamine (EA cells), leads to alterations in several physiological properties including cell separation (Tomasz, 1968, *Proc Natl Acad Sci USA* **59:** 86–93). Identical changes including chain formation and loss of autolytic properties can also be induced by adding up to 2% choline chloride to the growth medium (Briese and Hakenbeck, 1985, *Eur J Biochem* **146:** 417–427). Choline has been shown to inhibit the LytA pneumococcal autolysin (an *N*-acetylmuramoyl-L-alanine amidase) by preventing its attachment to wall teichoic acids (Giudicelli and Tomasz, 1984, *J Bacteriol* **158:** 1188–1190). In addition, it has been shown that the pneumococcal surface protein A (PspA) is anchored to the choline residues of the membrane-associated lipoteichoic acids (Yother and White, 1994, *J Bacteriol* **176:** 2976–2985).

Pneumococcus synthesizes several proteins that recognize and bind to the choline residues of the teichoic and lipoteichoic acids through a specialized domain, the choline-binding domain (ChBD) (Sánchez-Puelles *et al*., 1990, *Gene* **89:** 69–75). ChBD is built up of six or more well-conserved motifs, each about 20-amino-acid residues long (García *et al*., 1988, *Proc Natl Acad Sci USA* **85:** 914–918; García *et al*., 1998, *Microb Drug Resist* **4:** 25–36). Currently, there is an increasing interest in the study of pneumococcal genes coding for proteins that possess ChBDs. These proteins have been demonstrated to participate in a series of important biological functions such as cell adhesion and division. For many years, we have studied the molecular structure and biological role of the lytic enzymes of pneumococcus and its bacteriophages (López *et al*., 1997, *Microb Drug Resist* **3:** 199–211). These enzymes, which exhibit different chemical substrate specificities (i.e. lysozymes, amidases and glucosaminidases), display a modular organization in which the catalytic domain and the ChBD are located at the N- and C-terminal moieties of the protein respectively.

It is likely that a defective autolytic system might explain the physiological alterations leading to chain formation in *S. pneumoniae.* Recently, two independent experimental approaches have generated pneumococcal mutants that do not require choline or analogues for growth (Severin *et al*., 1997, *Microb Drug Resist* **3:** 391–400; Yother *et al*., 1998, *J Bacteriol* **180:** 2093–2101). These mutants form long chains when growing under choline-free conditions, and it has been claimed that the lack of an active LytA amidase, the main pneumococcal lytic enzyme, could be responsible for impaired cell separation at the end of cell division. Nevertheless, previous studies have demonstrated that the primary biological consequences of the *lytA* gene deletion were the formation of small chains (six to ten cells) and the absence of lysis in the stationary phase of growth (Sánchez-Puelles *et al*., 1986, *Eur J Biochem* **158:** 289–293; Ronda *et al*., 1987, *Eur J Biochem* **164:** 621–624) (Fig. 2A). A recent study of ABC-type Mn permease-defective mutants of pneumococcus also reports chain formation in the stationary phase of growth (*psaC* and *psaD* mutants) or the appearance of small conglomerates or simply aberrant morphology (*psaA* and *psaB* mutants) (Novak et al., 1998, *Mol Microbiol* **29:** 1285–1296). Nevertheless, a gene involved in the formation of long chains is not yet known in pneumococcus.

The fact that most bacteria possess several lytic enzymes makes it difficult to determine the precise physiological role of these enzymes. Overcoming this limitation requires experiments with well-defined, single, or perhaps multiple, mutants with altered peptidoglycan hydrolase. Using a previously published procedure to characterize the pneumococcal glucosaminidase (García *et al*., 1989, *Biochem Biophys Res Commun* **158:** 251–256), we identified, by SDS–PAGE, four protein bands with apparently strong choline binding affinity. One of these bands was excised from the acrylamide gel, and the N-terminal amino acid sequence was found to be Ser-Asp-Gly-Thr-Trp-Gln-Gly. This sequence was compared with the translated version of the partial nucleotide sequence of the *S. pneumoniae* genome (ftp://ftp.tigr.org/pub/data/s_pneumoniae), and a perfect match was found with an internal peptide of a gene product. This gene (hereafter designated *lytB*), located in the 10 373 bp contig no. 4117, was polymerase chain reaction (PCR) amplified, sequenced and preliminarily characterized (accession no. AJ010312). The putative 76.4 kDa LytB protein (658 amino acid residues) displays a modular organization different from all the ChBD proteins described previously in the pneumococcal system (Fig. 1A). Furthermore, this enzyme contains a 23-amino-acid-long, cleavable signal peptide (predicted M_r of the processed protein 73 800), as in the case of the *S. pneumoniae* LytC lysozyme, a new murein hydrolase recently

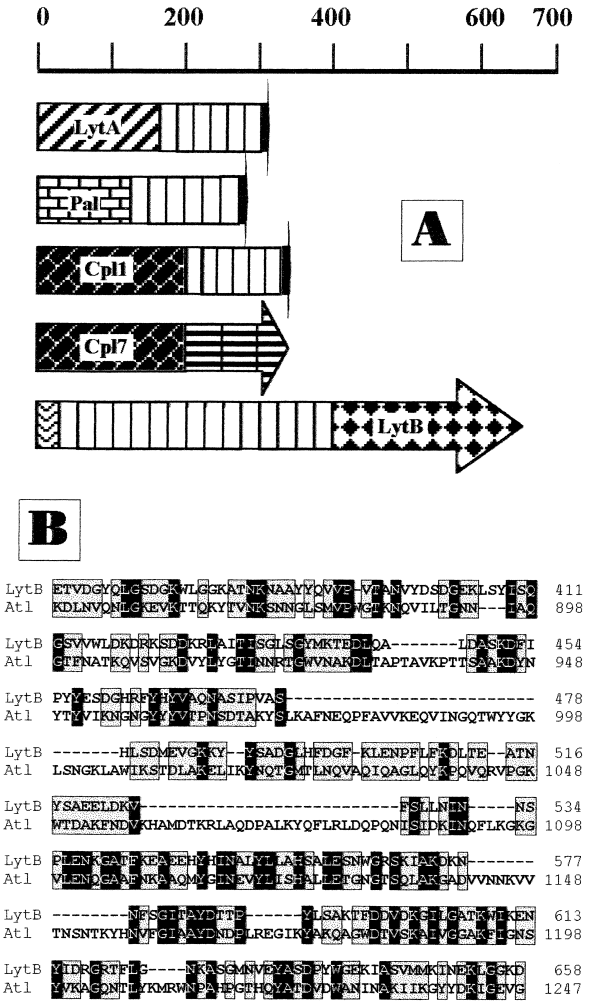

Fig. 1. Domain organization and sequence comparison of LytB and other murein hydrolases.
A. Comparison of the most relevant structural characteristics of several peptidoglycan hydrolases of *S. pneumoniae* and its bacteriophages. represent the domain containing the active site of the enzyme. represents the binding domain with the variable number of motifs. represents the signal peptide. The scale at the top represents the amino acid number.
B. Computer-generated alignment (BESTFIT) of the LytB domain putatively containing the active site with the glucosaminidase domain of the *Staphylococcus aureus* Atl autolysin (Oshida *et al.*, 1995, *Proc Natl Acad Sci USA* **92:** 285–289). Residues on black boxes indicate identical amino acids, whereas conserved substitutions are shown in dotted boxes. Numbers at the right correspond to the amino acid positions.

identified in our laboratory (manuscript in preparation). It appears that LytB has a completely different organization from that described for other enzymes of this type (Fig. 1A) (López *et al.*, 1997, *Microb Drug Resist* **3:** 199–211), because the order of the ChBD, with its 15 repeated motifs, and the catalytic domains is reversed in LytB. Sequence comparison suggests that LytB might be a glucosaminidase (Fig. 1B), possibly that previously studied biochemically in our laboratory (García *et al.*, 1989, *Biochem*

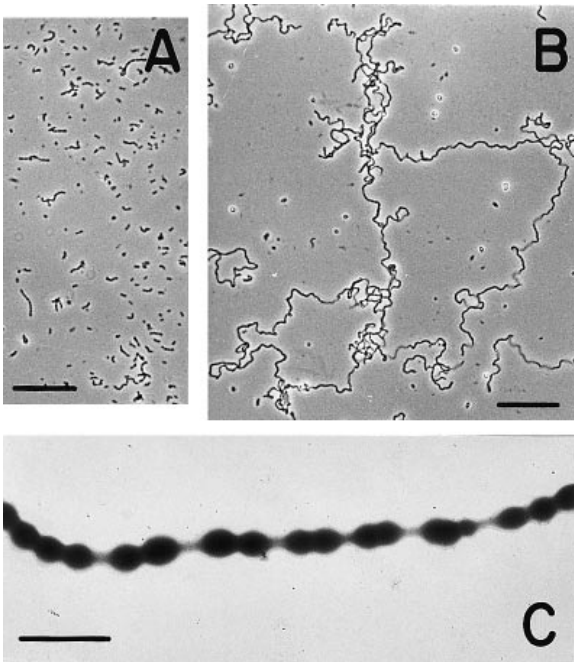

Fig. 2. Morphology of M31/*lytB* mutant. Chain formation of M31/*lytB* as examined by phase-contrast (B) and transmission electron microscopy (C). The parental strain M31 is also shown (A). In (A) and (B), the bar represents 25 μm. In (C), the bar represents 2 μm.

Biophys Res Commun **158:** 251–256). Most interestingly, the construction of a *lytB* pneumococcal mutant strain (R6B strain) by insertion–duplication mutagenesis resulted in the formation of long chains directly demonstrating that LytB plays a fundamental role in pneumococcal cell separation at the end of cell division. Still longer chains (more than 100 cells) were observed when the *lytB* mutation was introduced into the amidase-deficient, Δ*lytA* mutant M31 (Sánchez-Puelles *et al.*, 1986, *Eur J Biochem* **158:** 289–293) (Fig. 2), which confirms that the LytA amidase contributes in a moderate way to cell separation, as pointed out above. Cells within the chain are held together by thin filamentous material that seems to be an extension of the polar cell walls, as reported previously for EA cells (Tomasz *et al.*, 1975, *J Supramol Struct* **3:** 1–16) (Fig. 2C). In remarkable contrast to the EA cells, the recently described *psa* mutants and the choline-independent strains that exhibit the formation of chains in pneumococci, the R6B mutant did autolyse in the stationary phase as expected for a *lytA*$^+$ strain. Furthermore, pneumococci undergo rapid lysis upon exposure to sodium deoxycholate (a classical test for identifying *S. pneumoniae*) by triggering the LytA activity. As expected, the R6B mutant was as sensitive to lysis by sodium deoxycholate as the wild-type pneumococcal strain (data not shown).

It is conceivable that the evolutionary pressure operating in the *in vivo* environment of pneumococci selects

against the loss of autolytic activity, as the breaking up of bacterial chains might improve the chances of the pathogen's survival in its encounter with phagocytic cells (Tuomanen *et al*., 1988, *J Infect Dis* **158:** 38–43). Separation of fully divided cells is not essential for further growth, division and survival of the bacteria, as also suggested by the normal growth rate exhibited by the R6B mutant described here. However, chaining does inhibit the dissemination of daughter cells and, thus, could affect distribution, for example towards nutrients or away from inhibitors, or the dissemination of the microorganism during an infection. Therefore, cell separation could influence pathogenesis (Berry *et al*., 1989, *Infect Immun* **57:** 2324–2330).

In summary, the observations reported here show the fundamental role of LytB in the terminal step that leads to the separation of the daughter cells in *S. pneumoniae*. A detailed molecular and biochemical approach to the study of the LytB protein by cloning and expression of the *lytB* gene will contribute in the future to provide interesting data to explain fully the physiological conditions underlying the intriguing impaired separation of the pneumococcal strains that form chains. Moreover, the new LytB murein hydrolase described here could be an attractive specific target susceptible for the development of new selective antibacterial drugs.

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Operator sequences for the regulatory proteins of restriction modification systems

Sir,

For some type II restriction modification (RM) systems, it has been shown that transcription of the methylase gene (*M*) and the restriction endonuclease gene (*R*) is regulated by the control gene (*C*) product (Tao *et al*. 1991, *J Bacteriol* **173:** 1367–1375). In these systems, *C* is located directly upstream of *R*, and in most systems *M* is located divergently from *CR* (Fig. 1). The control element is a short (≈80 amino acids) protein containing a helix–turn–helix DNA-binding motif, distantly related to the well-known phage lambda cI regulator.

The DNA-binding sites from other proteins that bind with helix–turn–helix motifs (e.g. lambda cI and the LysR family of regulatory proteins) are usually 7–9 bp in size, of which only few bases directly interact with the protein. Most of these proteins form dimers, having a binding site of 14–18 bp with a dyad symmetry for at least the directly interacting bases. Based on sequence comparisons of the regions upstream of different *C* genes, two groups have proposed 'C-box' operator sequences of 12 bp and 18 bp respectively (Rimšeliene *et al*. 1995, *Gene* **157:** 217–219; Anton *et al*. 1997, *Gene* **187:** 19–27). The proposed C-box consensus sequences are unnecessarily large for a binding site of a monomeric form of the regulator. These sequences are also unlikely binding sites for a dimeric form of the regulatory protein because they lack dyad symmetry. In addition, control elements differing in the recognition helices of their respective helix–turn–helix motifs are unlikely to share identical operator sequences. Dissimilarity of the respective operator sequences is supported by the limited complementation of a *bamHIC* mutation by multicopy *pvuIC* and *smaIC*. In addition, a *pvuIC* mutation was better complemented by *bamHIC* than vice versa (Ives *et al*. 1995, *J Bacteriol* **177:** 6313–6315).

The *C* gene product exerts a negative influence on the transcription of *M* and a positive effect on *R* transcription (Rimšeliene *et al*. 1995, *Gene* **157:** 217–219). The operators for *M* and *R* might overlap for RM systems with divergent gene order. However, in RM systems in which the gene order is not divergent, *M* and *C* should have separate and most likely identical, operator sequences (Fig. 1).

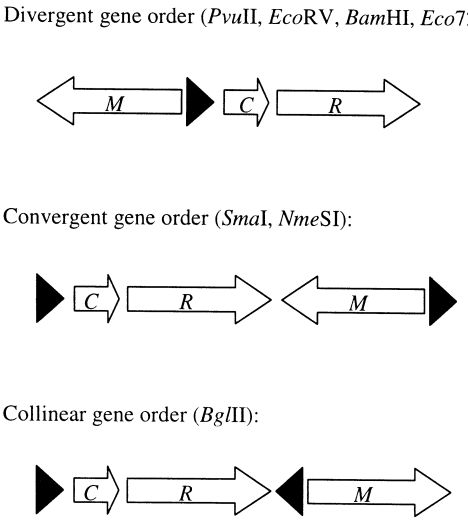

Fig. 1. Schematic representation of the gene order in RM systems that contain a gene for a control element. Arrows indicate the positions of the restriction endonuclease (marked *R*), methylase (marked *M*) and control element (marked *C*) genes. Black triangles indicate the position and orientation of the here-proposed control element operators. Data were taken from GenBank entries X16458 (*Sma*I), U49842 (*Bgl*II), X52681 (*Pvu*II), X00530 (*Eco*RV), X55286 (*Bam*HI), X76192 (*Mun*I) and from Rimšeliene *et al*. 1995, *Gene* 157: 217–219 (*Eco*72I). The sequence of the *Nme*SI system will be submitted to GenBank.

Fig. 2. Alignment of the upstream regions of *C* and *M* of the *Sma*I, *Nme*SI and *Bgl*II RM systems. The upstream regions of the *M* genes are inverted. Distance to the (proposed) start codons of the respective genes are given, except for *nmeSIM*, where the putative start codon is given in bold typeface. The identical DNA sequences are shaded. The earlier proposed C-boxes are underlined.

Therefore, we analysed the sequences of the *Sma*I and *Nme*SI (recently characterized in our laboratory) systems and the *Bgl*II system, in which *CR* and *M* are orientated convergently and collinearly respectively. For each of these three RM systems, we found identical sequences in opposite orientation in front of the open reading frames of *C* and *M* (Fig. 2). These sequences partially overlap the previously suggested C-boxes upstream of the *C* genes. Given the aforementioned considerations, these sequences are more likely to contain a 7–9 bp control element binding site within them than the previously proposed C-boxes. Noticeably, the recognition helices of SmaIC and NmeSIC are identical, and their sequences in Fig. 2 share the 7 bp sequence TGCTACT. The putative operators in front of *R* and *M* are in opposite orientations, suggesting that the orientation of the operator determines whether the regulator exerts upregulation or downregulation. This implies that one operator would be sufficient when the gene order is divergent (Fig. 1).

In conclusion, based on sequence data of RM systems with non-divergent gene order, we postulated operator sequences that are consistent with the dual regulatory role of control elements in RM systems in general.

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Comments on 'Classification and genetic characterization of pattern-forming *Bacilli*' *Mol Microbiol* (1998) 27: 687–703

Sir,

Rudner *et al*. (1998, *Mol Microbiol* **27:** 687–703) showed that the *Bacillus subtilis* strain 168, which has been reported to form branching and changing colony patterns by Ben-Jacob *et al*. (1992, *Physica A* **187:** 378–424), coexists with group I *Bacilli*. Thus, characteristic colony morphotypes and their variable properties reported by Ben-Jacob *et al*. have been revealed not to be the outcome of adaptive multicellular behaviour of *B. subtilis*. Certainly, with our *B. subtilis* strains, the tip-splitting (T) morphotype interconvertible to the chiral (C) morphotype demonstrated by Ben-Jacob *et al*. has not been observed.

Rudner *et al*. also mentioned the possible coexistence of group I *Bacilli* in the strains used in our studies on fractal colony formation and raised questions on our morphogenic model [e.g. diffusion-limited aggregation (DLA) growth in a nutrient diffusion field; Matsushita and Fujikawa, 1990, *Physica A* **168:** 498–506; Matsuyama and Matsushita, 1992, *Appl Environ Microbiol* **58:** 1227–1232]. However, our fractal growth model was proposed by studies on colony morphogenesis of many species of bacteria. On page 699 of the article by Rudner *et al*., they cited our article (Matsuyama *et al*. 1989, *FEMS Microbiol Lett* **61:** 243–246) as a possible example of group I *Bacilli* coexistence. In that paper, we described the fractal nature of a two-dimensionally growing pattern of a *Serratia marcescens* colony, but no *B. subtilis* strains were used in the experiments. In fact, from the centre to the growing front, the giant fractal colony exhibited the characteristic red trait of *S. marcescens*, unambiguously showing what species of bacteria was generating the fractal pattern. We also showed that bacterial ability to form the fractal pattern is kept even in flagellaless immotile *S. marcescens* mutants.

B. subtilis OG-01 and its flagellaless mutants were able to develop a fractal giant colony similar to a *S. marcescens* colony by cultivation on a nutrient-poor hard agar medium for 3–4 weeks (Matsushita and Fujikawa, 1990, *Physica A* **168:** 498–506; Ohgiwari *et al*. 1992, *J Phys Soc Jpn* **61:** 816–822). On page 699 of their article, Rudner *et al*. insisted on similarity between our *B. subtilis* fractal colony and the T morphotype made by their group I *Bacilli* (a coexistent in *B. subtilis* 168). However, the T morphotype is reported to emerge after 24–48 h of cultivation (Rudner *et al*. 1998, *Mol Microbiol* **27:** 687–703), and bacterial cells are reported to be moving in the T morphotype (Ben-Jacob

et al. 1994, *Fractals* **2:** 15–44). On the contrary, our *B. subtilis* is immotile on a hard agar, forms a small round colony over a short incubation time and exerts a gradual expanding colony growth over an extended long incubation time. Thus, in spite of similarity in colony morphology, colony expanding and pattern generation mechanisms seem to be quite different between our strains and group I *Bacilli*. The contribution of cell motility in colony growth may be a differentiating factor between our fractal colony and their T morphotype. So, we are wondering whether or not their T morphotype was also generated by immotile mutants of group I *Bacilli* after 24–48 h cultivation.

Cell motility-independent fractal giant colony formation has been reported with several species of Gram-negative rods (*Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, etc.) by Matsuyama and Matsushita (1992, *Appl Environ Microbiol* **58:** 1227–1232). The growth rate of a giant fractal colony (3–4 weeks for growth to the size of about 50 mm in diameter) is very much consistent with the diffusion time of nutrient molecules over an area with the same size. We also confirmed the critical effect of nutrient diffusion field in the fractal colony morphogenesis of immotile cells by showing screening and repulsion effects among extending colony branches of *B. subtilis* and *Salmonella* spp. (Matsushita and Fujikawa, 1990, *Physica A* **168:** 498–506; Matsuyama and Matsushita, 1992, *Appl Environ Microbiol* **58:** 1227–1232). Preceding our experiments, these nutrient diffusion field effects had been predicted by computer simulation of a DLA model by Meakin (1986, *J Theor Biol* **118:** 101–113).

In contrast to strains used by Ben-Jacob *et al*., bacteria taken from any part of a *B. subtilis* OG-01 colony grown on a nutrient-poor hard agar generate the same pattern. So, it was impossible to subdivide our strain. Rudner *et al*. described that coexisting group I *Bacilli* in *B. subtilis* 168 stocks are different from genuine *B. subtilis* by possessing β-galactosidase activity. Our strain OG-01 was β-galactosidase negative. On the same Xgal medium, our stock *Bacillus circulans* ATCC 4513 and *Bacillus alvei* ATCC 6344 were β-galactosidase positive, as reported by Rudner *et al*. (1998, *Mol Microbiol* **27:** 687–703). Thus, our *B. subtilis* OG-01 is different from group I *Bacilli* with respect to β-galactosidase activity, and T/C morphotypes generating cell motility.

There are many colony patterns formed by immotile bacteria. These colony patterns may be the outcome of various unidentified factors. However, we are thinking that the DLA-like process is fundamental for most bacterial colonies growing in a nutrient diffusion field over a long incubation time. So, whether our strain OG-01 strictly belongs to *B. subtilis* or not is not critical for our morphogenic model. Even so, we thought that it is necessary to examine 16S rRNA gene sequences of the strain OG-01. In the paper by Rudner *et al*., 16S rRNA gene sequencing was carried out by the method reported by Suzuki and Yamasato (1994, *FEMS Microbiol Lett* **115:** 13–18). So, we asked Dr Suzuki (Department of Applied Biological Science, Science University of Tokyo, Japan) to work on our request. Strain OG-01 cells taken from a point-inoculated centre and an expanding fringe of a representative fractal colony were examined for 16S rRNA gene sequences. Similarity values among 1506 nucleotide sequences of the OG-01 centre and fringe (DDBJ accession numbers AB018484 to AB018485) and the sequence of *B. subtilis* 16S *rrnA* gene (Ogasawara *et al*. 1983, *Nucleic Acids Res* **11:** 6301–6318) were all shown to be 99.9%. Thus, our OG-01 strain and cells actually being engaged in a fractal colony morphogenesis certainly belong to *B. subtilis*.

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We thank Tomonori Suzuki for his analysis in molecular taxonomy.

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Response to Matsuyama and Matsushita's MicroCorrespondence regarding our paper 'Classification and genetic characterization of pattern-forming *Bacilli*' *Mol Microbiol* (1998) 27: 687–703

Sir,

In our study, we used a molecular genetic approach to classify and define pattern-forming *Bacilli*. Using 16S rDNA sequence analysis, Southern hybridizations and phenotypical characterizations, we showed that strains reported by Ben-Jacob *et al*. (1992, *Physica* **238:** 181–197) to form *T*, *C* and *V* patterns were not *B. subtilis*, but instead represent new species belonging to group I *Bacilli*. We showed further that commonly used laboratory strains of *B. subtilis*, a group II species, can coexist as mixed cultures with group I *Bacilli*, and that the latter go unnoticed when grown on frequently used substrates.

In our discussion, we had suggested that a bacterium isolated from laboratory food, reported as *B. subtilis* by Matsushita and colleagues, resembled instead a group I *T*-morphotype for the following reasons: (i) its pattern

shape on nutrient-poor hard agar (Fig. 5 in Matsuyama and Matsushita, 1993, *Crit Rev Microbiol* **19:** 117–135; and Fig. 2 in Fujiwaka, 1994, *FEMS Microbiol Ecol* **13:** 159–168) looked nearly identical to the *T*-morphotypes we characterized in nutrient-poor hard agar (our Fig. 1); (ii) many strains of *B. subtilis* that we studied (our Table 3) did not form elaborate patterns on nutrient-poor hard agar; instead they remained compact; (iii) purified *T*-morphotypes and purified *B. subtilis* strains took 24–48 h to form patterns on nutrient-poor hard or nutrient-rich soft agar respectively. In contrast, *T*-morphotypes that emerged from *B. subtilis* populations took 3–4 weeks to form patterns on nutrient-poor hard agar. The strain described by Matsuyama and Matsushita (1993, *Crit Rev Microbiol* **19:** 117–135) took 3–4 weeks.

Since our publication, we have been in contact with Matsuyama and Matsushita and have learned of the following additional information, some of which is included in their MicroCorrespondence: (i) their strain OG-01, isolated from laboratory food, was characterized as *B. subtilis* by the Tokyo Metropolitan Research Laboratory of Public Health; (ii) OG-01, like *B. subtilis*, is β-galactosidase negative, unlike *T/C*-morphotypes and other group I *Bacilli*, which are β-galactosidase positive (we assume on Xgal plates); (iii) the 16S rDNA isolated from an OG-01 pattern is 99.9% homologous to the *B. subtilis* 16S *rrnA* gene. These results indicate that our original suggestion, that their strain OG-01 was a group I *Bacilli*, is wrong, and their strain is *B. subtilis*. However, as none of the above information was available or published at the time we wrote our study, understandably the last two items were performed in response to our study, the reasoning for our original suggestion was made on valid grounds at the time.

Matsuyama and Matsushita have also noted a citation error in our manuscript. On p. 699, column 2, line 12, we cited Matsuyama *et al*. (1989, *FEMS Microbiol Lett* **16:** 243–246) when referring to studies that began more than 10 years ago in their laboratory on pattern formation in *B. subtilis*. This reference should have been Fujikawa and Matsuyama (1989, *J Phys Soc Jpn* **58:** 3875–3878) and was included earlier in the same sentence on line 11. The same correction applies to column 2, line 22. We thank them for pointing this out to us and our readers.

Matsuyama and Matsushita stated that we raised questions on their DLA model for describing fractal colony growth (i.e. patterns). We did not raise questions about the actual model. We raised questions about the hypothesis of bacterial mutations instructed by 'cybernators' (Ben-Jacob, 1997, *Contemp Phys* **38:** 205–241) and, more recently, of bacterial learning (Matsushita, 1997, In *Bacteria as Multicellular Organisms*. Shapiro and Dwarkin, eds. Oxford University Press, p. 375) to describe what seem to be irreversible changes in the bacterium's pattern-forming properties. In the former case, we proved that it was instead the result of studying another species. As indicated in our article and by Matsuyama and Matsushita in their MicroCorrespondence, whether or not a strain is *B. subtilis*, the actual mathematical models used to describe its pattern of growth are still valid. We ask, however, that caution be used or genetic characterization performed before invoking mutation or learning as a property of bacterial pattern formation. Such assumptions may affect how mathematical models are designed.

In closing, we are intrigued by the interesting paradox that OG-01 presents. As far as we know, it is the only *B. subtilis* species known to form a pattern on the nutrient-poor hard agar concentrations used, resembles a *T*-morphotype in shape and takes 3–4 weeks to generate.

Perhaps there are small genetic differences between the relatively isogenic strains of presumably wild-type OG-01 and commonly used *B. subtilis* strains. All the *B. subtilis* strains we used were either derivatives of 168T or wild-type NCTC3610 (our Table 3). Genes that generate such a difference may prove to be critical in the machinery that governs pattern formation. Matsuyama and Matsushita raised questions of motility as a possible difference between OG-01 and what we found, and this could represent a genetic difference. Although, we did not systematically address motility in our study, we have noted that our *T/C* morphotypes and group II *Bacilli* have flagella, seen under low EM 16 000× magnification (R. Rudner, unpublished observations). Moreover, microscopic observations by Ben-Jacob *et al*. (1992, *Physica* **238:** 181–197; Ben-Jacob, 1997, *Contemp Phys* **38:** 205–241 in hard agar) and Matsuyama and Matsushita (1993, *Crit Rev Microbiol* **19:** 117–135), Matsushita (1997, In *Bacteria as Multicellular Organisms*. Shapiro and Dwarkin, eds. Oxford University Press, p. 375; in soft agar) have shown that bacterial cells forming patterns are confined to fluid envelopes. We only observed pattern formation when *T/C* morphotypes or *B. subtilis* were moving on hard or soft agar respectively (unpublished observations), unlike OG-01, which is immotile on hard agar, but still forms a pattern. Other possibilities still exist. Slight unapparent differences in nutrient agar properties could account for the discrepancies between our and Matsushita and Matsuyama's studies. This alternative possibility can only be tested in side-by-side comparisons of OG-01 with other known *Bacilli*. We eagerly extend an offer to Matsuyama and Matsushita to perform such a test with OG-01. Whatever the outcome, the results should prove useful in generating insight into the mechanisms and evolution of bacterial multicellular pattern formation.

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