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Helical *Bacillus subtilis* macrofibers: Morphogenesis of a bacterial multicellular macroorganism

(cell–cell interactions/cell surface organization/growth/forces in structure morphology/helical geometry)

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ABSTRACT **Helical bacterial macroorganisms have been produced by the selection of appropriate *Bacillus subtilis* mutants and the establishment of specific growth conditions. Threadlike fibers ranging in length to approximately 1 cm are produced in fluid culture by the parallel association of many division-suppressed filaments in helical arrangement. A more open ball-like structure of complicated woven architecture may also be produced. Macrostructure morphology is regulated by genetic, physiological, and nutritional factors. The pitch angle of surface filaments in helical macrofibers varies as a function of macrofiber diameter, indicating a flexible response of individual cell surfaces to the forces responsible for helical morphology. Three classes of mutants have been obtained that are concerned with helix directionality: (*i*) mutants that form only left-handed helix macrofibers, (*ii*) mutants that form only right-handed helix macrofibers, and (*iii*) conditional mutants able to form either left- or right-handed helix macrofibers depending upon nutritional environment. Aggregate structures containing both left- and right-handed macrofibers have been obtained by coculturing appropriate mutants. In addition to providing information on the organization of the bacterial cell surface, this new system offers unique and unusual opportunities to study cell–cell interactions, primitive morphogenesis, and the properties of a multicellular bacterial form.**

One of the fundamental differences between bacterial populations as conventionally studied and higher organisms is the organization of cells in space. Usually, bacterial fluid cultures consist of dispersed random assortments of cells at all stages of the cell division cycle. There is no spatial continuity between parent and progeny cells. In contrast, multicellular organisms develop, largely as a result of cell surface interactions, organized associations of cells which serve either structural or functional roles in the life of the organism as a whole. The range of higher-order phenomena studied in developmental biology is based upon such organization. To explore analogous phenomena in bacteria, one needs a system in which the normal dispersive mechanisms do not operate and in which the cell surfaces adhere to one another. A system with these properties has recently been discovered. Fluid cultures of special *Bacillus subtilis* mutants are capable of forming highly organized multicellular structures. The multicellular forms progress through a complex series of morphologies during growth that is reminiscent of the "life-cycles" found in higher multicellular organisms. The addition of new cells and cell rearrangement are important features of morphogenesis in this bacterial "macrobe." It is now realistic to investigate a number of fundamental biological principles in a bacterial model system, principles that hitherto could only be studied in higher organisms. The present publication will show why this is so.

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MATERIALS AND METHODS

Bacterial multicellular macrobes have been cultured in fluid in two different media: one, a rich complex medium described previously for culture of strain B1S (1), referred to as TB; the other, an enriched minimal medium commonly used to induce the competent state for genetic transformation in *B. subtilis* (2), referred to as S1 in this publication. The complex medium was supplemented with uracil, the minimal medium with uracil and methionine (20 μg/ml for each supplement).

The culture method employed consisted of static drops approximately 0.1 ml in volume distributed on the inside of a plastic petri dish lid maintained in the inverted position with the petri dish base used as a cover. Twelve single-drop cultures were grown in each petri dish. After inoculation the cultures were incubated in a moist chamber at about 20°. Such cultures are conveniently observed with a low-power stereoscopic microscope (7–40× magnification) that employs illumination from below. Selection for cloning on the basis of macrostructure morphology was performed using this system. larger volume cultures consisting of 5 ml of either medium dispensed in either plastic petri dishes (100 mm × 15 mm) or glass screw-cap test tubes (16 mm × 150 mm) were employed for studies of macrobe life cycles and for maintenance of macrobes. The test tube cultures were incubated slanted so as to maximize surface area. For microcinematography, cultures were grown in quartz flat capillary tubes (viewing path 0.2 mm, outside dimensions 0.6 mm × 2.4 mm, Vitro Dynamics, Rockaway, NJ), incubated at 20° on a phase-contrast microscope stage.

All multicellular macrobes discussed in this publication were derived from the original helix-producing strain of *B. subtilis* (B1S) described in an earlier publication (1). Derivatives of the type that produce right-handed helix macrobes in either TB or S1 were obtained by repeated selection and cloning of superior structures in TB. The conditionally reversible class of helix handedness mutants was obtained by a similar regime using S1 medium beginning with a B1S macrostructure produced in TB.The class of mutants able to produce only left-handed helix macrobes was obtained by selection and cloning one of the conditionally reversible mutants (RHX) in TB medium. With the exception of the original B1S mutant, all the macrobe-producing derivatives are essentially asporogenic. These mutants have been maintained by: (*i*) vegetative transfer of macrostructures in fluid culture, (*ii*) repeated transfer on semi-solid medium of the same composition as used in fluid culture, and (*iii*) lyophilization (representatives of each upon rehydration were found to perpetuate helical macrobe growth).

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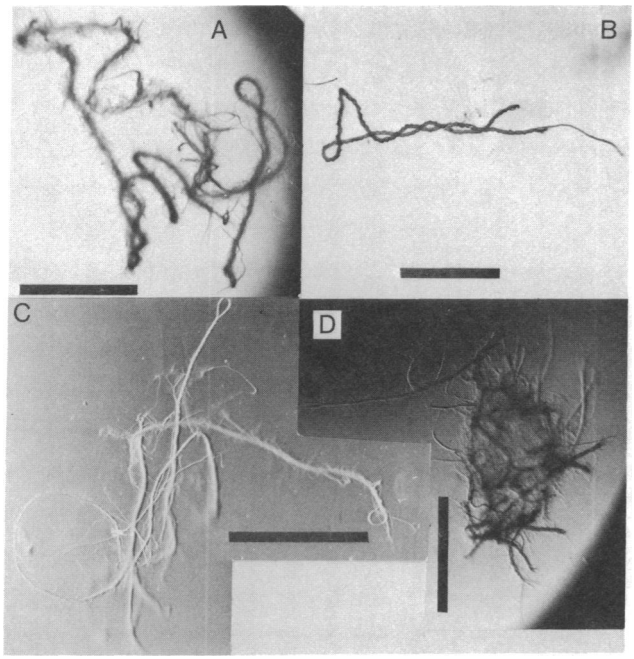

FIG. 1. Macrofibers of *B. subtilis*. Drop cultures were grown at 20° and photographed directly *in situ*. *A* and *B* show left-handed helix structures produced by mutant C6. *C* and *D* show right-handed helix structures produced by mutant OR-11. Medium composition: S1 for *A* and *C*, TB for *B* and *D*. Bars: *A* and *C*, 1 mm; *B* and *D*, 0.5 mm.

Inoculum size and the physiological condition of cells used to initiate macrobe production are critical factors. Excellent structures have been obtained in three ways: (*i*) young colonies produced by overnight incubation at 20° of a streak on either TB or S1 agar were used as an inoculum source, (*ii*) macrostructures, produced in fluid culture either transferred intact or disrupted into fragments were found to be suitable inocula, and (*iii*) in later stages of growth macrobes liberate long cells into the culture medium. These cells also give rise to excellent macrobe structures.

Light microscopy techniques have been described (3).

Measurements of helix dimensions and other macrobe parameters were obtained from either phase contrast micrographs or low-magnification macrophotographs. The latter were obtained using an Aristophot assembly consisting of a 35-mm Leica camera body fitted with an extendible bellows to which either an f = 25 mm or f = 35 mm lens was attached. The measuring equipment has been previously described (1).

Dynamic models were explored using $\frac{3}{16}$ inch (0.48 cm) internal diameter × $\frac{1}{16}$ inch (0.16 cm) wall thickness amber latex rubber tubing.

RESULTS

Examples of helical multicellular macrobes are shown in Fig. 1. These structures were photographed in the original drop cultures in which they were produced. All are visible to the naked eye as white threadlike or clumplike structures. The size and morphology of structures shown are typical of those found in drop cultures inoculated with the random number of cells that are shed from a toothpick tapped in the medium drop and incubated at 20° for 18 to 24 hr. With the exception of the macrostructure, the drops remain clear, indicating that the cells have not grown throughout the medium as in a conventional bacterial culture, but rather have remained together in the form of a large multicellular structure. A number of structural details are evident in Fig. 1. First, it is clear that the macrobes are built of a hierarchy of helices. Each structure consists of a number of fibers helically intertwined, which in turn join other fibers, progressively increasing the diameter of the major fiber. In addition, the fibers can fold back upon themselves and helically wrap together, thereby producing loop ends and correspondingly larger diameter fibers. The individual fibers that join one another need not be of equal dimensions either in width or length. In regions where subfibers of grossly different dimensions unite, the macrostructure accommodates the individual entities into a tightly organized cohesive unit. Model building has indicated that, to achieve this, appreciable spatial reorganization of the individual cells is required. Evidence will be presented below that addresses this point.

Other features evident in Fig. 1 are: (*i*) there is no gross morphological difference between left- (Fig. 1 *A* and *B*) and right- (Fig. 1 *C* and *D*) handed helix macrofibers; (*ii*) distinct macrofibers are formed in both S1 (Fig. 1 *A* and *C*) and TB (Fig. 1 *B* and *D*) media; (*iii*) at later stages of growth, macrofibers become reorganized into more open (Fig. 1*A*), eventually ball-like structures. Certain mutants when grown in TB go through a stage similar to that shown in Fig. 1*D*, consisting of a ball-like center from which large macrofibers project. Time-lapse films of such structures indicate that growth is accompanied by helical rotation of the projecting fibers, many of which are eventually drawn into the surface of the ball. The direction of helical rotation observed is correlated with the handedness of the helix macrofiber produced.

The cellular architecture of helical macrofibers is revealed by phase-contrast microscopy as shown in Figs. 2 and 3. Fig. 2 illustrates the manner in which double-strand helices may join one another to produce uniform helical fibers of larger diameter. Tracing the individual strands in this figure, as well as in flexible rubber models built to simulate such structures, reveals that to accommodate one another the geometrical properties of each double helix must undergo rearrangement, particularly with respect to the pitch angle of the helix. Fiber growth in diameter is accomplished by the continued association of individual cellular strands, as well as by the joining of helical macrofibers to one another or the folding back of a macrofiber upon itself. In all cases the forming structure incorporates its

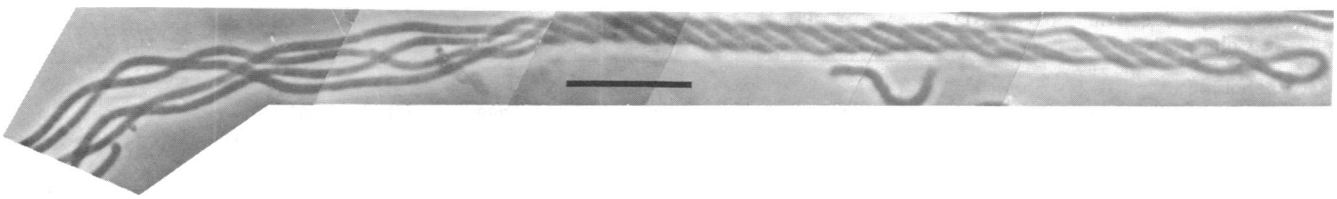

FIG. 2. Four-stranded helical fiber of *B. subtilis*. Right-handed helix structure produced in TB by mutant B1S, illustrating the close packing of two double stranded helices. Bar = 10 μm.

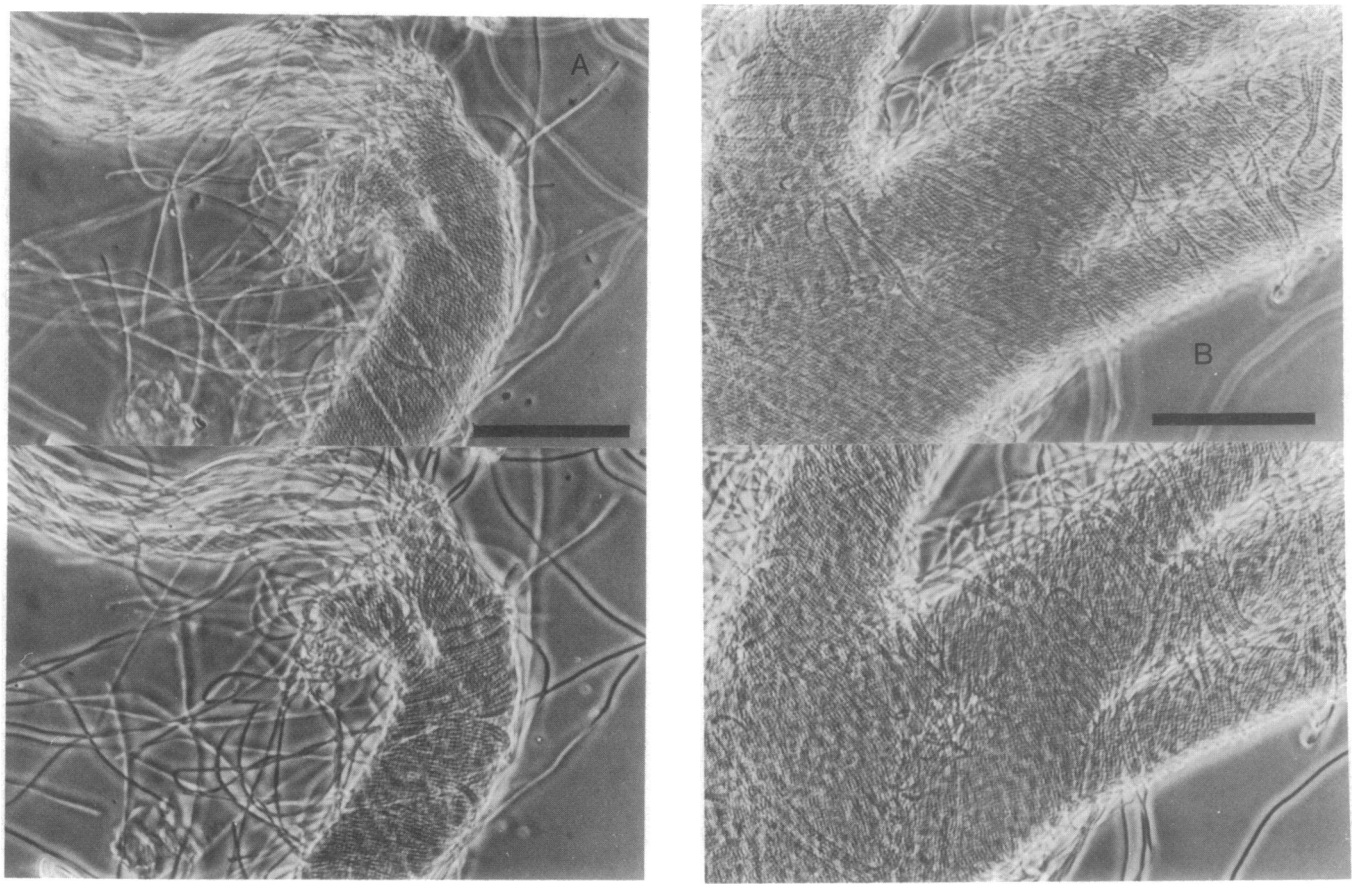

FIG. 3. Phase-contrast micrographs of *B. subtilis* macrofibers. Mutant RHX cultured (*A*) in S1, (*B*) in TB. In each pair the upper figure focuses on the uppermost layer of the cylinder, the lower figure focuses on the lowermost layer. Bars = 40 μm.

substructures into the larger helix by a process involving cellular rearrangements and helix adjustments analogous to those shown in Fig. 2 at a simpler level of complexity. Larger structures shown in Fig. 3 illustrate the tight packing arrangement of cells in helical macrofibers and the details of large fiber organization. The pitch angle of surface filaments is seen to be related to fiber diameter. To quantitate this relationship, micrographs of many fibers of different sizes were measured. The results shown in Fig. 4 illustrate that, as fibers increase in diameter, the pitch angle of surface filaments approaches 90° with respect to the long axis of the fiber. If this geometrical plan is followed to its limit, therefore, a theoretical maximum fiber diameter is defined because 90° is incompatible with helical structure. The largest fibers actually observed thus far have pitch angles in the range of 75°.

Fig. 3 illustrates, in addition, the simple manner of determining helix handedness in macrofibers. By focusing on the uppermost and lowermost surfaces of fibers, the surface filaments are seen as running from either lower left to upper right or vice-versa, along fiber length. Fig. 3*A* is a left-handed helix macrofiber. This technique was used to score large numbers of macrofibers produced by representative mutants that belong to the following three classes: (*i*) those that produce right-handed helix macrofibers, (*ii*) those that produce either right- or left-handed helix macrofibers depending upon the medium they are grown in, and (*iii*) those that produce left-handed helix macrofibers. The data in Table 1 illustrate these categories and demonstrate an unusual pattern of clonal inheritance with respect to helix handedness in the RHX mutant. In this conditionally reversible mutant, the proportion of left- to right-

Table 1. Heritability of helix direction in *B. subtilis* macrostructures

Mutant:	B1S				RHX				D5			
Fluid medium structure produced in:	S1		TB		S1		TB		S1		TB	
Colony origin:	TB	S1	TB	S1	TB	S1	TB	S1	TB	S1	TB	S1
Phenotype												
Right-handed helix	79	266	346	415	0	0	288	23	0	0	1	3
Left-handed helix	0	0	0	2	439	246	93	371	162	257	256	202
Total structures examined	79	274	346	420	440	246	440	437	162	260	257	207
Totals	1119				1563				886			
Grand total	3568											

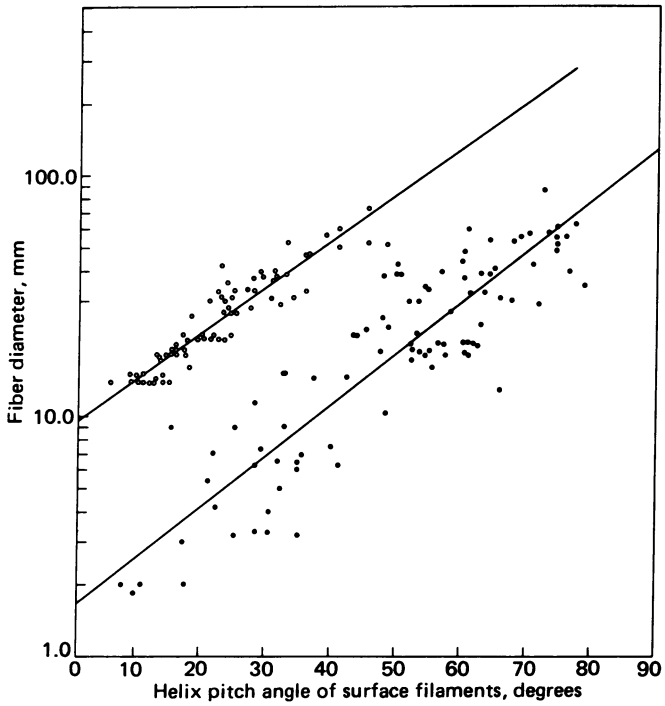

FIG. 4. The relationship of surface helix pitch angle to fiber diameter in *B. subtilis* macrofibers, and in models of similar geometry. Measurements were taken from phase-contrast micrographs of three right-handed-helix-producing mutants (6, OR-11, and RTD), two reversible helix direction mutants (RHX and 571133), and one left-handed helix mutant (C6). Both S1- and TB-grown fibers were included and measurements from both upper surface focus and lower surface focus were used. Scale: 1 mm on micrograph = 0.95 μm actual fiber dimension (●). Amber latex rubber tubing models were assembled using double-stranded helix subunits of the same initial pitch angle and helix direction. The subunits were assembled by wrapping in the same helix direction. Each assembly was measured by viewing through a supported glass plate raised so as not to touch or distort the cylindrical structure (○). Regression line slopes are 0.020 (●) and 0.019 (○).

handed structures produced in TB medium is strongly influenced by the previous medium on which the cells were grown. The fact that no right-handed helix structures were found in S1 medium suggests that the RHX mutant is not simply a mixture of cells belonging to the two other categories. Recently time-lapse films have documented the direct conversion of an individual RHX fiber from left- to right-handed macrostructure, following transfer from S1 to TB medium. The heritable influence of previous growth environment on helix direction in the RHX mutant is reminiscent of a crystal–seed phenomenon. The mechanism of this influence at the level of the bacterial cell surface isn't understood currently.

We have observed on numerous occasions the ability of macrofiber-producing strains to form their structures encrusted upon a contaminating cotton fiber in the drop culture. These observations prompted attempts to induce right- and left-handed helix-forming mutants to grow together into a single complex macrostructure. A number of combinations were attempted, several of which were successful in the sense that large aggregate structures arose which when serially transferred blind (without reference to their cellular composition) were able to perpetuate both right- and left-handed helix structures. One of these organisms is illustrated in Fig. 5. Fibers of both kinds are seen to enter the aggregate, where they eventually disorganize into individual filaments. On the exterior, left- and right-handed macrofibers retain their properties. These observations suggest that either (*i*) macrofibers perpetuate clonally within aggregates or (*ii*) cells of like handedness can recognize one another and sort during fiber morphogenesis.

DISCUSSION

The highly ordered multicellular macrobe produced from *B. subtilis* provides an opportunity to gain new insight into biological processes at several levels. The multicellular form may be viewed as a primitive organism, complete with its own unique life cycle. In this context questions may be asked about the structure of the organism, its morphogenesis and decay, and the factors that regulate these processes. Clearly we are dealing with a helical structure comprised of a hierarchy of helices, the limits of which appear to be defined by geometrical constraints of helix structure itself. The construction of helical macrofibers requires the parallel association of long, division-suppressed cells. Time-lapse films have familiarized us with the outlines of this process. Individual single filaments of different clonal origins as well as double-stranded helical filaments may join to growing structures. When cell surface contact is achieved, a helical torque associated with growth appears to force close packing of the filaments into a helical fiber. Once the process is begun, continued growth, which at the individual cell level is confined to cylinder elongation, may be transformed to growth of macrofiber diameter by a process of folding and branching. The films also indicate that growth in length of structures ranging from double-stranded helix units to large multistranded macrofibers is accompanied by a rotational turning of the entire structure. This finding is in accord with a prediction made earlier that bacterial surfaces are helically structured in a manner that necessitates rotation with elongation. The helical morphology of the macroorganism is interpreted therefore as a ramification of helical organization of the individual cells that comprise the structure.

If indeed the morphology of this macroorganism reflects the cell surface organization of the individual cells from which it is made, then several observations concerning the multicellular form must be incorporated into our view of cell surface organization. For example, the change from macrofiber to open ball-like structure must reflect either a change in cell surface organization or, perhaps, a cessation or slowing of growth accompanied by a relaxation of helical torque. More striking is the ability of mutants such as RHX to convert from a helix of one direction to that of the opposite. A change of this magnitude would necessitate a corresponding reorganization of the cell surface helix phase. If the cell surface were made of subunits one could visualize a mechanism similar to that described for helical phase changes in bacterial flagella (4, 5) to account for the observed analogous cellular transition. At this time it is impossible, however, to make any definitive statements concerning the architecture of the *B. subtilis* cell wall vis-a-vis substructure organization.

The adhesive properties of the cell surfaces required for macrobe assembly are not unique aspects of the particular mutants described in this publication. A brief survey conducted in collaboration with D. Karamata (University of Lausanne, Switzerland) of other division-suppressed *B. subtilis* mutants as well as other related *Bacillus* species has revealed that helical macrofibers can be produced by many different strains. In addition, similar phenomena have been described in *B. mycoides* (now considered a variety of *B. cereus*) in the early 1900s (see ref. 6, for example). Thus the highly selected fiber producing strains discussed above are perhaps more efficient and produce more uniform macrobes but they are not completely different from other related bacteria. Rather, it appears that

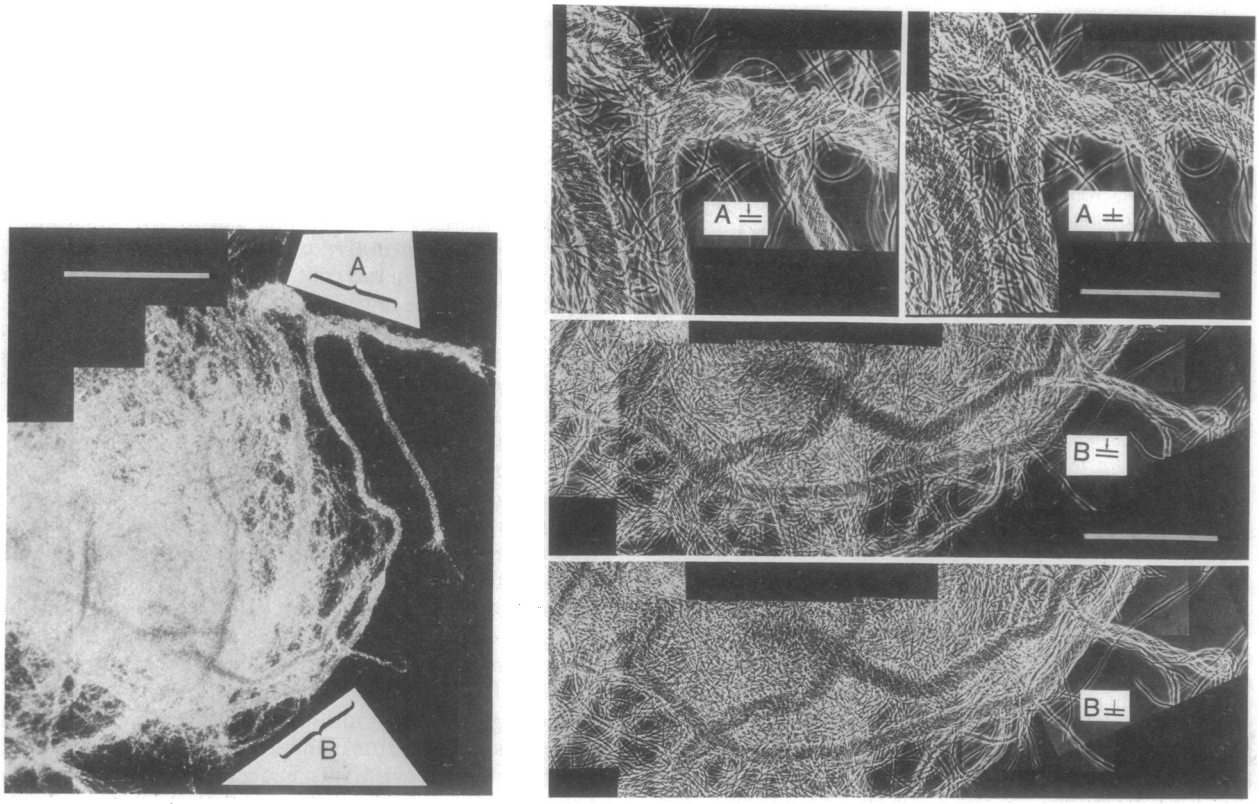

FIG. 5. An aggregate *B. subtilis* macroorganism consisting of both right- and left-handed helix macrofibers. The low-magnification view (*Left*) shows part of a large structure cultured in S1 medium. Bar = 200 μm. The regions designated A and B are shown also at higher magnification (*Right*). The arrows indicate focus on either the upper or the lower surface of the cylindrical macrofibers. Bars = 100 μm.

the cultural conditions and division suppression are key features in fostering cell surface adhesion and in keeping together the products of growth as required for multicellular macroorganism development. The helical property observed in all cases appears to reflect a fundamental helical cellular architecture and growth plan as originally proposed (1).

The association of both left- and right-handed helix macrofibers into an aggregate organism is the first of our attempts to create an organism of increased structural and developmental complexity. We have found that such aggregates are reasonably stable even in the absence of intentional selection. The forces responsible for morphogenesis in these complex forms invite further studies. We hope that these inquiries will also be pertinent to the ways in which cell interactions contribute to morphogenesis of higher organisms. In plants, for example, certain cell surfaces have been shown to grow in a helical pattern just as our model predicts for bacterial cells (7). In addition, numerous examples of plant organization based upon helical geometry are known (8). In animal cells it has recently been discovered that the long processes that grow from retinal neurite explants do so in a helical manner. In the neurite system the geometry of growth may be important in establishing the proper *in vivo* cellular contacts in the brain.

It appears therefore that helical geometry is important at levels of organization in the biological world ranging from macromolecular through subcellular, cellular, and even organismal structure. There must be some fundamental physical principles that underlie the presence of this particular architectural plan in these diverse systems. In 1950 the physicist H. R. Crane attempted to apply some simple physical principles to explain aspects of biological growth, particularly the assembly of smaller subunits into larger structures (9). Crane predicted "... any structure which is straight or rod-like when seen at 'low magnification' is probably a structure having repetition along a screw axis. At somewhat greater magnification such a structure would be expected to reveal a helical, zigzag, or banded appearance. To imagine a straight structure which did not owe its straightness to the cause just given would require the assumption of a highly fortuitous combination of angles between successive sections." Could it be as simple as this?

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