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Induction of Coordinated Movement of *Myxococcus xanthus* Cells

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Rhythmically advancing waves of cells, called ripples, arise spontaneously during the aggregation of *Myxococcus xanthus* into fruiting bodies. Extracts prepared by washing rippling cells contain a substance that will induce quiescent cells to ripple. Three lines of evidence indicate that murein (peptidoglycan) is the ripple-inducing substance in the extracts. First, ripple-inducing activity is associated with the cell envelope of sonically disrupted *M. xanthus* cells. Second, whole cells, cell extracts, or purified murein from a variety of different bacteria are capable of inducing ripples. In contrast, extracts prepared from *Methanobacterium* spp. which contain pseudomurein instead of typical bacterial murein fail to induce ripples. Third, four components of *M. xanthus* murein, *N*-acetylglucosamine, *N*-acetylmuramic acid, diaminopimelate, and D-alanine, are able to induce ripples. Ripples produced by aggregating cells have a wavelength of 45 μm and a maximum velocity of 2 μm/min. Both of the multigene systems that control gliding motility appear to be required for rippling, and all known mutations at the *spoC* locus eliminate both rippling and sporulation.

Myxobacteria, such as *Myxococcus xanthus*, are procaryotes with a multicellular life cycle. When starved, tens of thousands of cells aggregate into a center where they construct a fruiting body whose form is species specific. As the fruiting body of *M. xanthus* matures, the rod-shaped vegetative cells transform into spherical, dormant myxospores. When feeding, thousands of cells move in close proximity to each other forming a coherent "swarm." Swarm cells are not bound rigidly to each other and are free to move individually, but the entire mass of cells migrates as a unit (7, 12, 13). How do myxobacterial cells coordinate their behavior in swarming and fruiting?

During time-lapse photographic studies of swarming and fruiting (12, 13), Reichenbach discovered a pattern of multicellular movement, which is illustrated in Fig. 1. Reichenbach observed the formation of series of equidistant ridges separated by troughs in a dense assemblage of fruiting bacteria. The whole system of ridges and troughs appeared to move like trains of water-like ripples emanating from the center of the cell mass. Because of their resemblance to ripples on a water surface, we refer to the ridges and troughs as "ripples." Reichenbach also noted that rippling itself waxed and waned (12, 13). Our measurements on Reichenbach's time-lapse films showed that a period of approximately 20 min for movement of individual ripples was superimposed on a longer period of about 5 h for the waxing and waning of the whole field of ripples. Rippling is characteristic of all members of the genus *Myxococcus* and has also been observed in *Stigmatella aurantiaca* (12, 13). Although ripples almost always appear during the aggregation phase of fruiting body formation, as illustrated in Fig. 1, their relation to development has not previously been studied.

The wavelength of ripples corresponds to about 10 cell lengths, and their long-range order seems to imply coordination of movement between cells in adjacent ridges as well as between neighboring cells in the same ridge. We began to analyze this process when we discovered that an extract of rippling cells could induce rippling in quiescent cells. This paper describes the isolation and identification of a ripple-inducing substance from those cell extracts. The relation between rippling and cell movement and between rippling and fruiting body development is also examined.

MATERIALS AND METHODS

Bacterial strains and phages. Table 1 lists the strains of *M. xanthus* used, their genotypes, and their origins. The myxophage Mx8cp2 was used for all transductions (10). *Escherichia coli* C3 was obtained from J. Kobori, *Salmonella typhimurium* LT2 was from G. Weinstock, and *Micrococcus luteus* and *Bacillus subtilis* were from M. Dworkin. Lyopholyzed cells of

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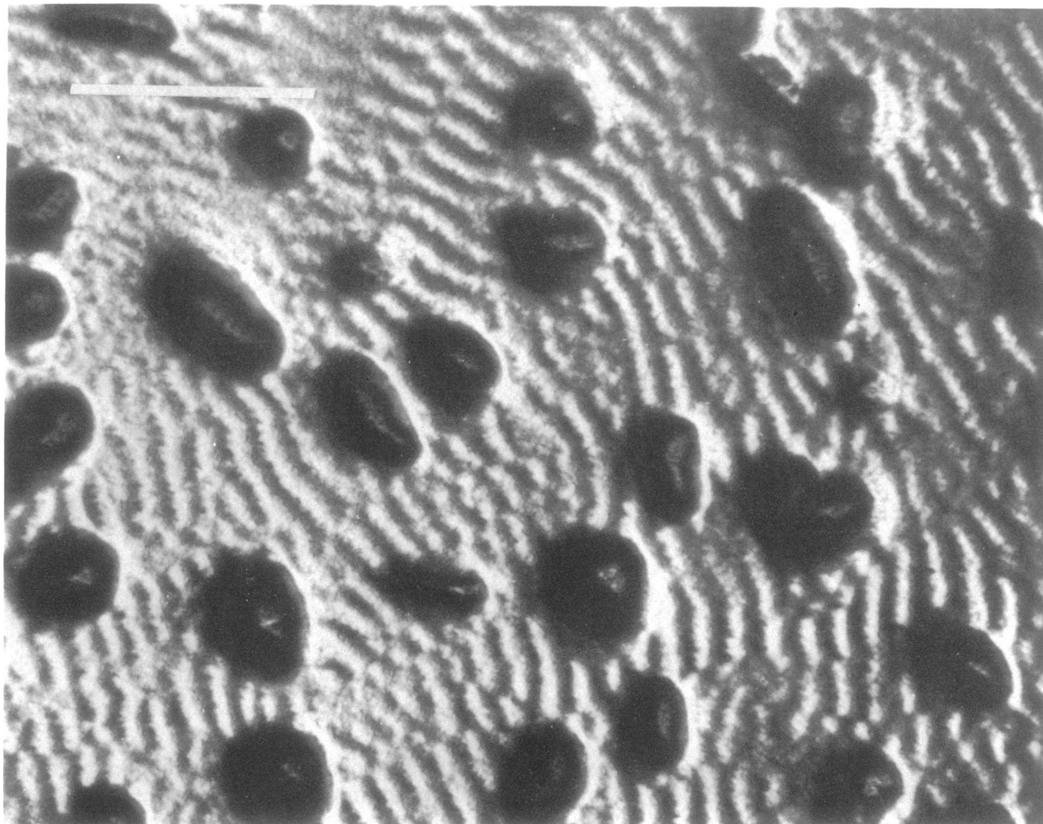

FIG. 1. Ripples generated during development of fruiting bodies. Cells of strain DK1622 were spotted on CF agar at a density of 1.6×10^7 cells per cm^2 and incubated at 32°C for 18 h. The dark masses are immature fruiting bodies. Bar, 400 μm.

Methanobacterium thermoautotrophicum and *Methanobacterium bryantii* were a gift from R. Wolfe. Purified *Corynebacteria poinsettiae* peptidoglycan was a gift from H. Perkins.

Media. Cells were grown vegetatively in CTT broth, which contains 1% Casitone, and ½ CTT, which contains 0.5% casitone, and induced to develop on CF agar (1, 2).

Phage growth and transduction. Growth, assay, and transduction with Mx8 have been described previously, as has the use of the transposon Tn*5* as a selectable marker for moving linked genes from strain to strain (8, 13).

Preparation and assay of ripple-inducing activity. Ripple-inducing extracts were prepared from *M. xanthus* and a variety of other bacteria by disrupting washed, log phase cells with sonic oscillation for 60 s (Mullard; 5 μm peak to peak). This treatment was sufficient to kill >99.99% of the cells. Broken cells were lyophilized and stored at −20°C. The material was resuspended at 40 mg/ml in distilled water and dispersed by sonic oscillation.

Extracts were assayed for ripple-inducing activity by one of two different procedures. In the agar assay, exponentially growing DK1622 cells were spread on CTT agar or ½ CTT agar at a density of 8×10^6 cells per cm^2 or onto CF agar at a density of 1.6×10^7 cells per cm^2, and 5 μl of the extract was applied in a small spot on top of the cells. Extracts were diluted in twofold steps, and each dilution was tested in duplicate. After 24 h at 33°C, plates were examined under a dissecting microscope for ripples.

The second assay utilized the submerged culture technique of Kuner and Kaiser (9). Exponentially growing cells were diluted in fresh CTT broth to 2×10^7 cells per ml, and 2 ml was pipetted into a 5-cm-diameter plastic petri dish (Falcon Plastics; no. 1006), sealed, and incubated at 33°C for 20 h. During this incubation the cells settled to the bottom, adhered, and then grew to form a cohesive mat. The CTT broth was drawn off and replaced with an equal volume of distilled water for 15 min, and then the distilled water was replaced with 10 mM morpholinepropanesulfonic acid (pH 6.8), 1 mM $CaCl_2$, 0.2% (wt/vol) Casitone, and additional supplements as mentioned. In the Kuner procedure for fruiting body development in submerged cultures, CTT broth is replaced by a solution of morpholinepropanesulfonic acid and $CaCl_2$. The addition of a low level of Casitone (0.2 to 0.5%) prevents fruiting body development and spontaneous rippling, but allows rippling to be induced by cell extracts and related compounds as described below. Supplements were tested at a series of twofold dilutions, and each dilution was tested in triplicate. After

TABLE 1. Myxobacterial strains[a]

Strain	Motility	Sporulation	Source or derivation
DK101	A^+ S^- *sglA*		(5)
DK836	A^+ S^+		Soil isolate (J. Wood)
DK898	A^+ S^+		Soil isolate (J. Wood)
DK1050	A^+ S(Ts) *sgl*(Ts)		F. Murillo
DK1212	A^- S^+ *aglJ*		(5)
DK1216	A^- S^+ *aglQ*		(5)
DK1217	A^- S^+ *aglB*		(5)
DK1221	A^- S^+ *aglG*		(5)
DK1253	A^+ S^- *tgl*		(5)
DK1300	A^+ S^- *sglG*		(5)
DK1622	A^+ S^+		D. Morandi
DK1696	A^- S^- *cglC sgl*		D. Morandi
DK1820	A^+ S^- *sglB*		(5)
DK2121	A^- S^- *cglB sgl*		D. Morandi
DK2608	A^- S^+ *cglC*		Mx8 (DK1970) × DK1622 → Km^r [A^-]
DK2612	A^- S^+ *cglE*		Mx8 (DK2728) × DK1622 → Km^r [A^-]
DK2614	A^- S^+ *cglF*		Mx8 (DK2730) × DK1622 → Km^r [A^-]
DK2616	A^- S^+ *cglB*		Mx8 (DK2754) × DK1622 → Km^r [A^-]
DK2618	A^- S^+ *cglD*		Mx8 (DK1973) × DK1622 → Km^r [A^-]
DK2630	A^+ S^+	*spo-741* Ω1519	Mx8 (DK1525) × DK1622 → Km^r [Spo^-]
DK2631	A^+ S^+	Ω1519	Mx8 (DK1529) × DK1622 → Km^r [Spo^+]
DK2632	A^+ S^+	*spo-653* Ω1519	Mx8 (DK1529) × DK1622 → Km^r [Spo^-]
DK2634	A^+ S^+	*spo-731* Ω1519	Mx8 (DK1536) × DK1622 → Km^r [Spo^-]
MD2	A^+ S^- *sgl*		M. Dworkin

[a] Each independent insertion of the transposon Tn*5* is identified by an omega number that designates the insertion site. The bracketed symbol in the last column indicates the motility [A^-] or sporulation [Spo^+ or Spo^-] phenotype of the particular kanamycin-resistant transductant chosen.

the addition of the supplement, the plates were sealed, incubated at 33°C for 18 to 24 h, and then examined for ripples and fruiting bodies with a dissecting microscope.

Purification of peptidoglycan. Peptidoglycan of *M. luteus* was purified by a modified version of the Park and Hancock procedure (11). Cells were grown in CTT at 33°C with vigorous shaking and pelleted at 10,000 × *g* for 5 min. The pellet was suspended in 10% trichloroacetic acid on ice for 10 min and then centrifuged. Insoluble material was resuspended in 75% (vol/vol) ethanol and incubated at room temperature for 10 min. After centrifugation the pellet was resuspended in 10% trichloracetic acid and boiled for 15 min. The trichloroacetic acid-insoluble material was washed twice with 3 mM Tris-hydrochloride (pH 7.6) and incubated in 3 mM Tris with 1 mg of trypsin per ml at 37°C for 24 h. The soluble material was washed in distilled water and lyophilyzed. For use, the peptidoglycan was suspended in distilled water at 40 mg/ml and sonicated to yield a uniform suspension.

Scanning electron microscopy of ripples in submerged culture. Glass cover slips were washed in ethanol, flamed, and placed on the bottom of a plastic petri dish. Because *M. xanthus* produces slime in submerged culture which tends to obscure the outline of individual cells, the submerged culture procedure was modified to reduce the amount of slime. A 10-fold increase in the initial inoculum (2 × 10^8/ml) followed by a 2-h incubation period resulted in a mat of cells comparable to that produced by the standard procedure, but with less slime material. The plates were washed, and 0.5 mg each of *N*-acetylglucosamine, *N*-acetylmuramic acid, and D-alanine per ml was added. After 24 h at 33°C glutaraldehyde was added to a 2.5% final concentration for 30 min and replaced with 2% OsO_4 for an additional 30 min. The glass cover slips were transferred to a glass petri plate and dehydrated in increasing acetone concentrations (10, 25, 50, 75, 90, and 100% [vol/vol]). The cover slips were sputter coated with 200 nm of gold and examined in an ISI-40 scanning electron microscope.

Analysis of the propagation of ripples. Ripples were induced on medium containing glass beads and were photographed at intervals at 27°C. The negatives were printed on Kodalith ortho film type 3 (Eastman Kodak Co.; no. 2256), which produces a nearly transparent positive image. These photographs were superimposed with the glass beads as position markers, and the movement of ripples with time was recorded.

RESULTS

Demonstration of ripple-inducing factor in cell extracts. Extracts of *M. xanthus* induced rippling in a lawn of vegetative cells. Figure 2 demonstrates how the behavior of cells on agar was changed by addition of an extract prepared by sonically disrupting cells in the aggregation phase of development. The distribution of test cells 18 h after addition of extract (Fig. 2A) contrasted dramatically with that in Fig. 2B, which shows a parallel culture to which only buffer had been added. In the culture exposed to extract (Fig. 2A), cells accumulated in sets of

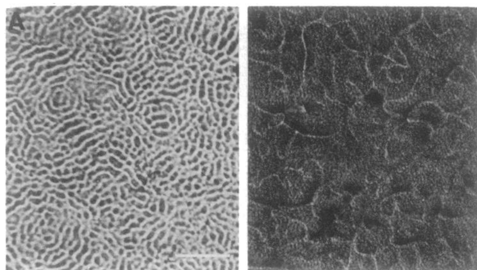

FIG. 2. Induction of ripples in growing cells by an extract of rippling cells. DK1622 cells were spread at a density of 2×10^6 cells per cm^2 on ½ CTT agar, and then (A) 10 μl of a sonic extract of rippling cells (10^{10} cells per ml) or (B) 10 μl of distilled water was spotted on top of the cells and incubated at 32°C for 18 h. Bar in panel A, 400 μm.

equally spaced ridges. The spacing between ridges in Fig. 2 was similar to that found during fruiting body development (Fig. 1). Fruiting bodies were not produced in Fig. 2 because the responding cells were growing in the presence of 0.5% Casitone (½ CTT agar). The development of fruiting bodies requires that cells be starved as in Fig. 1, but ripples can be induced in cells growing on low-nutrient media.

Ripple-inducing activity was also found in sonic extracts of vegetatively growing cells (DK1622 grown in CTT broth at 33°C to a density of 4×10^8 cells per ml). These extracts were adjusted to the same turbidity as the active extracts of developing cells, but there was a difference in the physical state of the active material. Developing cells (24 h at 33°C on CF agar), harvested by scraping them from the plate, suspending them in water, and then sedimenting them at $10,000 \times g$, released active material into the supernatant liquid. The cells, which sedimented at $10,000 \times g$, released more active material after sonic treatment. In contrast, vegetatively growing cells sedimented at $10,000 \times g$ from CTT broth released no active material to the supernatant; ripple-inducing activity sedimented with the cells and had to be released by sonic treatment.

To aid the chemical identification of ripple-inducing material, activity was quantitated by testing twofold serial dilutions of the extract until a no-ripple endpoint was reached. Table 2 shows the distribution of activity in fractions of an extract of growing cells, as determined by this endpoint assay. Although half of the activity from sonically disrupted vegetative cells was found in the insoluble (membrane) fraction and half was found in the soluble fraction, all of the activity in the resulting soluble fraction was sedimented at $100,000 \times g$, and almost all of the activity was precipitated by 40% saturated $(NH_4)_2SO_4$. Electron microscopic examination of the $100,000 \times g$ pellet and the ammonium sulfate precipitate revealed tiny membrane vesicles, approximately 0.1 μm in diameter, that appeared to have been sheared from the cells by the sonic treatment. These observations suggested the possibility that the ripple-inducing activity was associated with the cell envelope.

TABLE 2. Fractionation of activity from sonically disrupted cells[a]

Step	Procedure	% of initial activity in: Sediment	Supernatant
1	Sonically disrupted cells, sedimented 10 min at $10,000 \times g$	50	50
2A	Supernatant from step 1, precipitated by 40% saturated ammonium sulfate	44	6
2B	Supernatant from step 1, sedimented 30 min at $100,000 \times g$	50	0

[a] Strain DK1622 grown in CTT broth to 4×10^8 cells per ml was sedimented at $10,000 \times g$ for 10 min, suspended in water at 4×10^9 cells per ml, and broken by sonic oscillation. The fractions indicated were brought to equal volumes, diluted in twofold steps in water, and assayed on DK1622 on 1/2 CTT agar. Note that the total activity input in steps 2A and 2B is 50%.

Ripple-inducing activity was extracted from many different bacteria, including three gram-negative and three gram-positive species (Table 3). Ripple-inducing activity correlated with the type of cell wall present in the cells from which the extract was obtained. Extracts of bacteria containing murein (peptidoglycan) composed of

TABLE 3. Sources of ripple-inducing activity

Source	Type of cell wall	Ripples
M. xanthus[a]	Murein	+
E. coli[a]	Murein	+
S. typhimurium[a]	Murein	+
M. luteus[b]	Murein	+
B. subtilus[a]	Murein	+
C. ponsettiae[b]	Murein	+
M. thermoautotrophicum[a]	Pseudomurein	−
M. bryantii[a]	Pseudomurein	−
Crab shells[c]	Chitin	−
Wood[c]	Cellulose	−
S. cerevisiae[a]	Mannan	−

[a] Sonically disrupted cells were assayed with DK1622 on 1/2 CTT agar.
[b] Purified peptidoglycan was assayed as in *a*.
[c] Commercial preparations of cell wall polymers were assayed as in *a*.

N-acetylmuramic acid and D-amino acids were active. Purified murein from *Micrococcus luteus* and *Corynebacterium poinsettiae* was active. The pseudomureins from two species of *Methanobacterium* which contain L-amino acids instead of D isomers and *N*-acetylthalossauronic acid instead of *N*-acetylmuramic acid, were inactive (5, 6). Other nonmurein cell walls such as chitin, cellulose, and extracts of the yeast *Saccharomyces cerevisiae* were also inactive.

Ripple-inducing activity of murein components. A second assay for ripple-inducing materials that is suitable for soluble and highly diffusible molecules as well as insoluble ones is based on the submerged culture technique of J. Kuner. For this assay cells are grown on the bottom of a petri dish under a layer of liquid growth medium containing the substances to be assayed. Figure 3 is a photograph of a submerged culture with (A) and without (B) the addition of soluble murein components. Response in this assay was all or none; either ripples formed over the whole plate or they did not form at all, as if the phenomenon were highly cooperative. Table 4 lists the specific activities of single murein components and of mixtures of components. *N*-Acetylglucosamine was the only murein component that was effective alone, and its specific activity was only 1/10 that of murein; 10 times as much *N*-acetylglucosamine as murein was required to induce rippling. Three other murein components, *N*-acetylmuramic acid, D-alanine, and diaminopimelic acid, were found to enhance the activity of *N*-acetylglucosamine. Any pair or any triplet of the four components was active, but a mixture of all four had the highest specific activity, and the activity of the mixture was the same as purified *M. luteus* murein. The four active compounds are all components of *M. xanthus* murein (16).

The compounds shown in the lower part of Table 4 are designated inactive because they exhibited three properties: (i) failure to induce ripples alone, (ii) failure to induce ripples in pairwise mixtures with D-alanine, diaminopimelate, or *N*-acetylmuramic acid, and (iii) failure to increase the specific activity of *N*-acetylglucosamine when mixed with it. Certain components of *M. xanthus* murein are inactive by these criteria; L-alanine and D-glutamate, for example. Components of unusual mureins such as L-ornithine and D-glutamine were also inactive. Apparently the inductive mechanism is capable of discriminating between closely related compounds and even stereoisomers. For instance, L-alanine will not substitute for D-alanine, nor will L-lysine substitute for diaminopimelate. The stereospecificity of diaminopimelate has not yet been determined as the material tested was a mixture of the LL, DD, and *meso* isomers.

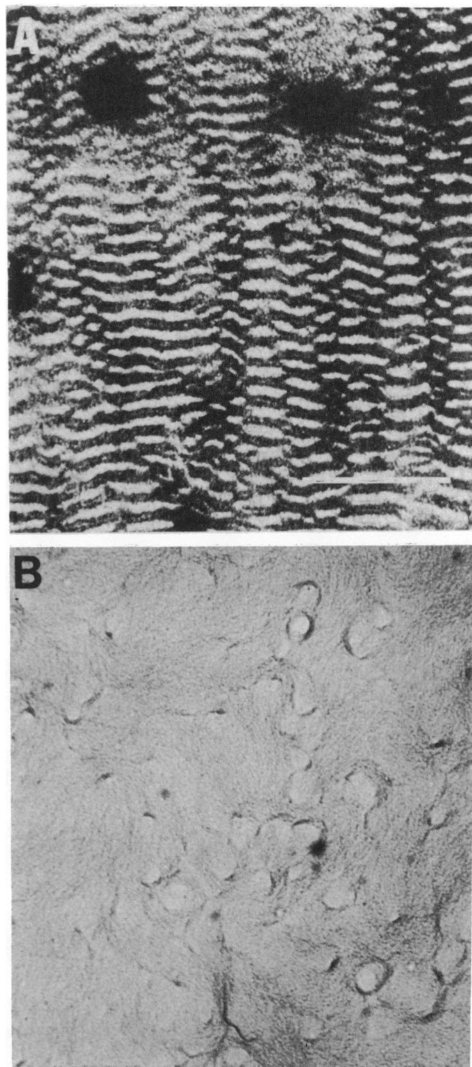

FIG. 3. Induction of ripples in submerged culture by components of murein. (A) Culture with 2.5 mM each *N*-acetylglucosamine, D-alanine, and diaminopimelate. (B) Culture with no additions. Strain DK1622 was treated in submerged culture as described in the text. Bar in panel A, 400 μm.

The rippling state was quite stable. After rippling had begun in submerged culture, it continued even when the inducing solution was replaced with a solution lacking inducers. In one such replacement experiment the cell layer was washed three times with fresh buffer, yet ripples were observed subsequently for at least 8 h. Moreover, vigorous agitation of the culture dish for several hours did not prevent or noticeably alter rippling. These experiments show that the

TABLE 4. Ripple-inducing substances

Substance added[a]	Sp act (U/mg)[b]		
	Alone	Combined with MurNAc or Dpm or DAla	Combined with GlcNAc
M. luteus murein	2.0		
GlcNAc	0.2		
MurNAc	<0.4		
DAla	<0.025		
Dpm	<0.025		
MurNAc + GlcNAc	0.7		
GlcNAc + DAla	0.9		
Dpm + DAla	0.9		
GlcNAc + Dpm	1.0		
MurNAc + Dpm	0.7		
DAla + MurNAc	0.9		
GlcNAc + MurNAc + DAla	1.0		
MurNAc + DAla + Dpm	0.6		
GlcNAc + DAla + Dpm	1.0		
GlcNAc + MurNAc + Dpm	0.5		
GlcNAc + MurNAc + Dpm + DAla	2.0		
L-Alanine	<0.025	<0.1	0.1
D-Glutamate	<0.025	<0.1	0.1
Pyruvate	<0.025	<0.1	0.1
L-Lysine	<0.1	<0.2	0.1
L-Ornithine	<0.1	<0.2	0.1
D-Glutamine	<0.1	<0.2	0.1
Glycine	<0.1	<0.2	0.1

[a] Abbreviations: GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; DAla, D-alanine; Dpm, DL-α-ε diaminopimelic acid.

[b] One unit of activity is the minimum amount that will induce rippling of DK1622 in submerged culture as described in the text. The minimum was determined by endpoint dilution with twofold dilution steps. Equimolar amounts were employed in the mixtures of two or more substances, and the specific activity is based on the total weight of all substances added.

signals used to propagate and to space the ripples are not affected by washing or agitating the cells.

Fruiting bodies were produced in submerged culture in response to added murein components (Fig. 3A). Each substance or mixture of substances listed in Table 4 that induced ripples also induced the formation of fruiting bodies provided the nutrient concentration was sufficiently low (0.2% Casitone). When the Casitone concentration was increased to 0.5%, ripples without fruiting bodies were induced by murein components. Thus, fruiting body development and rippling are both responses to the addition of murein components, but fruiting body development also requires lower mutrient levels.

Movement of cells in ripples. *M. xanthus* has two sets of genes, called system A and system S, that independently provide for cell movement (3, 4). System S by itself allows movement of cells only when they are near each other, whereas the A system by itself allows movement independent of the distance between cells. Wild-type cells have both systems active and are said to be fully motile or $A^+ S^+$. To test whether rippling depends on either of these motility systems, fully motile ($A^+ S^+$) strains and mutants ($A^- S^+$, $A^+ S^-$, and $A^- S^-$) defective in one or both systems were examined for their ability to ripple when murein was added (Table 5). All three fully motile ($A^+ S^+$) strains rippled; two of these are recent soil isolates of *M. xanthus* (J. Wood, unpublished data), and the third, DK1622, is a reconstructed parent of the rest of the strains in the table. All mutants lacking a functional A system failed to ripple. All but one of the mutants lacking a functional S system failed to ripple. The exception, MD2, is distantly related to DK1622, and its ripple response was evident only on occasion. Nonmotile mutants with defects in both systems failed to ripple. The mutants examined were defective at different loci in the A and S systems (Table 1). Thus, it appears that rippling is an organized movement requiring the activity of both A and S motility systems.

To examine the movement of individual ripples, glass beads were added as position markers, and fields of ripples were photographed at 5-min intervals. Each negative was used to print a positive transparency. A series of related transparencies were superimposed by using the pat-

TABLE 5. Relation between type of motility and rippling[a]

Strain	Motility phenotype	Ripples
DK836	$A^+ S^+$	+
DK898	$A^+ S^+$	+
DK1622	$A^+ S^+$	+
DK1050	A^+ S(Ts)	+
DK1212	$A^- S^+$	−
DK1216	$A^- S^+$	−
DK1217	$A^- S^+$	−
DK1221	$A^- S^+$	−
DK2608	$A^- S^+$	−
DK2612	$A^- S^+$	−
DK2614	$A^- S^+$	−
DK2616	$A^- S^+$	−
DK2618	$A^- S^+$	−
DK1253	$A^+ S^-$	−
DK1300	$A^+ S^-$	−
DK1820	$A^+ S^-$	−
DK101	$A^+ S^-$	−
MD2	$A^+ S^-$	±
DK1696	$A^- S^-$	−
DK2121	$A^- S^-$	−

[a] Strains were plated on CF agar at a density of 1.6 $\times 10^7$ cells per cm^2 and 0.2 mg of *M. luteus* cell walls was spotted on top of cells and incubated at 33°C for 24 h. Each test was repeated three times. ± indicates that some tests were positive and others were negative.

tern of glass beads to bring them into alignment. The progression of individual ripples was then followed, and measurements on them are summarized in Table 6 for three different conditions. Ripples produced spontaneous during fruiting on CF agar and ripples induced by murein in submerged culture had similar wavelengths, maximum velocities, and periods. During a 30-min interval about half of the ripples on CF and in submerged culture were observed to move. The ripples induced by murein on a medium with higher nutrient levels, ½ CTT agar, were quantitatively different in that their wavelength was greater, their maximum velocity was greater, and virtually all of them moved during a 30-min interval. The observed maximum velocities of ripple movement are comparable to the maximum rate of single cell movement (Crosby and Kaiser, unpublished data).

The movement of ripples induced in submerged culture was studied to determine how the ripples, and cells within the ripples, are coordinated. Figure 4B shows that the ridges are accumulations of cells and the troughs are regions of cell depletion. In addition, the scanning electron micrograph (Fig. 5A) shows three ridges that are thick with cells and slime oriented horizontally, and two troughs with fewer cells between them. Ridges were not continuous across a whole field of ripples, but were divided into a series of parallel tracks (clearly evident in Fig. 4 and also evident in Fig. 2 and 3). The time-lapse photographic experiments described above showed that ripples within the same track move synchronously, in phase, and in the same direction. Ripples in adjacent tracks move in opposite directions or are stationary during any 30-minute period of observation. The contrary motion of ripples in adjacent tracks helps to explain why there is no large-scale transport of cells from one part of a ripple field to another. Figure 4 shows that ridges in adjacent tracks are connected to each other. At the border between two tracks the ridges tend to divide laterally (viewed along the ridge line) into two parts with one part branching leftward to join a ridge in the adjacent track and the other part branching rightward to join the next ridge in the adjacent track. This branching pattern of connections between ridges in adjacent tracks suggests that cells may move from one track of ripples to an adjacent track through these connections.

Examination of the high-magnification view of three adjacent ridges (Fig. 5) shows that many

TABLE 6. Propagation of ripples[a]

Culture	Wavelength (μm)	Period (min)	Maximum velocity (μm/min)	% Moving ripples
CF agar[b]	45 ± 11.0 (19)	20.1	2.2	49
Submerged culture[c]	49 ± 8.3 (20)	21.0	2.3	45
1/2 CTT agar[d]	70 ± 8.2 (26)	18.9	3.7	99

[a] Ripples were photographed at 5-min intervals. The wavelength (λ) is the distance from crest to crest ± 1 standard deviation. The number of measurements is within parentheses. The period (n) is the length of time for a ripple to move one wavelength and in each case is the fastest time observed. Maximum velocity (V) is calculated from the equation $nV = \lambda$. Percent moving ripples was determined from photographs taken over a 30 min interval.

[b] Spontaneous ripples.

[c] Ripples induced by adding 0.5 mg of purified *M. luteus* murein per ml of culture.

[d] Ripples induced by spotting 5 μl of purified *M. luteus* murein (40 mg/ml) on top of the cell layer.

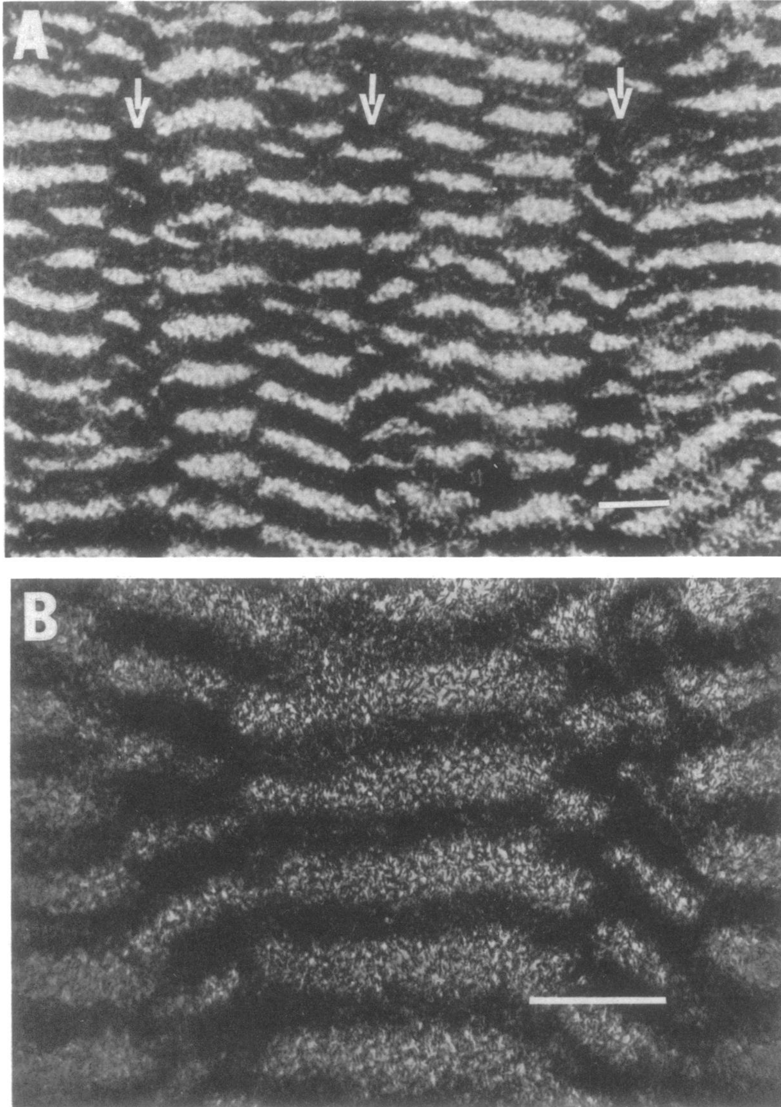

FIG. 4. Organization of ripples into tracks. (A) Ripples in adjacent tracks move in opposite directions and are separated by cross-over zones, some of which are indicated by arrows. Bar, 100 μm. (B) One track of ripples flanked by two cross-over zones. Individual cells are visible in the troughs between ripples. Bar, 100 μm. DK1622 cells in submerged culture plus 2.5 mM each *N*-acetylglucosamine, D-alanine, and diaminopimelate.

cells are oriented at similar acute angles to the ridge line. Although many cells are submerged in slime, which obscures their outline, the orientation of those cells on the surface is summarized in Fig. 6. A rather narrow distribution centered around 40° to the ridge line was observed for about 300 cells drawn from five different micrographs. Because *M. xanthus* cells generally move in the direction of their long axis (7, 13), this result suggests that the cells are moving at a 40° angle to the direction of ripple propagation. There is no corresponding peak of cells oriented at 140°, the mirror image of 40°. This asymmetry implies that cells distinguish between right and left. The angular distribution of cells in the trough between two ridges centered around 0° (= 180°) (Fig. 7).

Relationship of rippling to sporulation. The *spoC* locus was initially discovered because its activity is required for sporulation (1). Three mutant alleles map to this locus and are tightly linked to a Tn*5* insertion, Ω1519 (R. LaRossa,

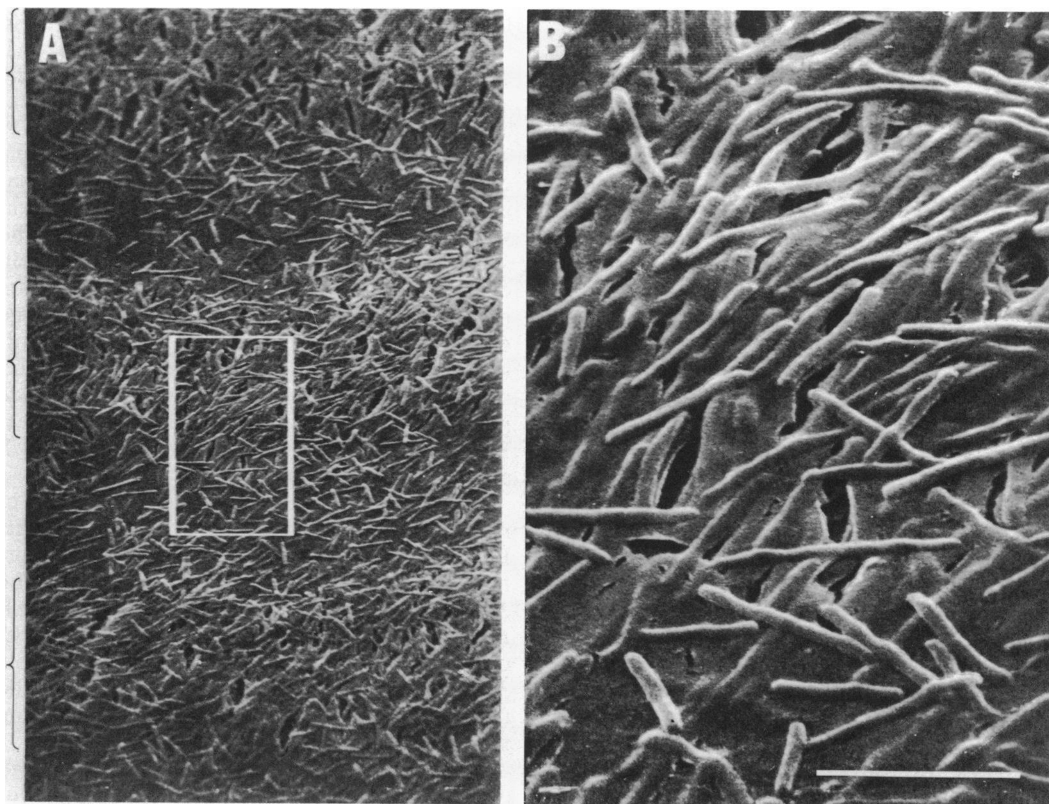

FIG. 5. Scanning electron micrograph of a portion of a field of ripples at low magnification (A) and high magnification (B) of the portion marked by a white rectangle. Individual rod-shaped cells are evident. Portions of three ridges running horizontally are indicated by the brackets on the left and are separated by two troughs (A). The ridges are accumulations of cells, many of which are buried in slime; the troughs are regions of cell depletion. Bar in panel B, 30 μm.

J. M. Kuner, D. Hagen, and D. Kaiser, C. Manoil, submitted). Each mutant allele was transduced into DK1622 to create a set of isogenic strains, and these strains were tested for their ability to ripple. All three *spoC* mutants were unable to ripple when induced with either

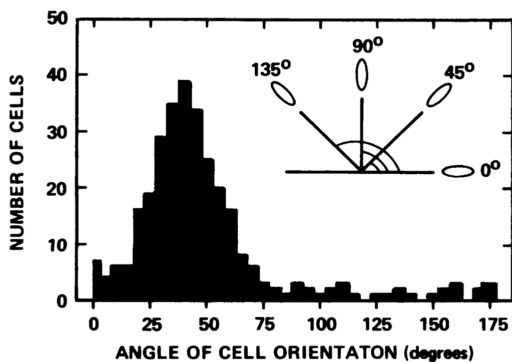

FIG. 6. Orientation of cells in ridges with respect to the direction of ridge movement. The angle between the long axis of each cell and the ridge line, measured clockwise (inset), is plotted versus the number of cells. A total of 303 cells from five microscope fields like that shown in Fig. 5 were measured.

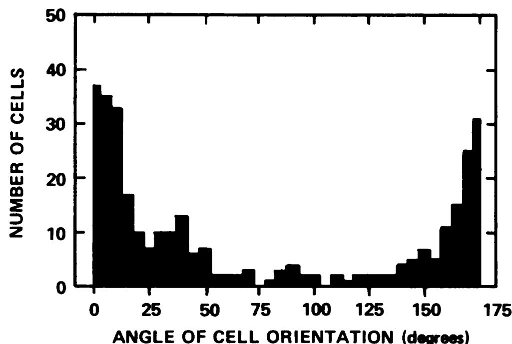

FIG. 7. Orientation of cells in troughs with respect to the direction of movement of adjacent ridges. The angular orientation is as defined in Fig. 6. A total of 323 cells from the same five microscope fields as Fig. 6 were measured. Note that 0° and 180° represent the same orientation since the two ends of a cell are indistinguishable.

TABLE 7. Rippling of nonsporulating mutants

Strain	Spores[a]	Murein-induced ripples[b]	Murein component-induced ripples[c]
DK2631	+	+	+
DK2630	–	–	–
DK2632	–	–	–
DK2634	–	–	–

[a] Cells were plated on CF agar at a density of 1.6×10^7 cells per cm^2, and spores were assayed microscopically after 5 days at 33°C.

[b] Submerged culture containing 0.5 mg of purified *M. luteus* murein per ml.

[c] Submerged culture containing 2.5 mM *N*-acetylglucosamine, D-alanine, and diaminopimelate.

Micrococcus murein or murein components, unlike their isogenic spo^+ relative, DK2631 (Table 7). This suggests that rippling and sporulation are dependent on a substance whose production is controlled by the *spoC* locus. Rippling and sporulation may therefore be related developmental events.

DISCUSSION

Ripples are ridge-shaped accumulations of cells moving in a synchronous, pulsating manner. A substance capable of inducing ripples has been isolated from the murein fraction of cell envelopes and appears to be peptidoglycan. Purified peptidoglycan, whole cells, or cell envelopes from a variety of bacteria with murein similar to that of *M. xanthus* are able to induce ripples. In contrast, the pseudomurein of two species of *Methanobacterium* which lack *N*-acetylmuramic acid, D-alanine, and diaminopimelate (5, 6) is unable to induce ripples. Chitin, cellulose, and extracts of *Saccharomyces cerevisiae* were also ineffective. In addition, four components of *M. xanthus* murein were able to induce ripples: the active components were *N*-acetylglucosamine, *N*-acetylmuramic acid, diaminopimelate, and D-alanine. *N*-Acetylglucosamine was the only component that was active alone, but its specific activity was 10-fold lower than that of purified murein. Any pair of the four active components was able to induce ripples or to raise the specific activity of *N*-acetylglucosamine. When all four of the components were added together, their ripple-inducing activity per gram was the same as that of purified murein.

Three different explanations for the similar specific activities of whole murein and of a mixture of murein components can be offered, apart from chance coincidence. First, murein might be hydrolyzed to its components by extracellular bacteriolytic enzymes. In fact three different enzyme activities have been observed to be secreted by *M. xanthus:* an endo-*N*-acetylglucosaminidase, an *N*-acetylmuramyl-L-alanine amidase, and a peptidase active on D-alanyldiaminopimelate (15). Although these enzymes would not be capable of hydrolyzing all intercomponent linkages in murein, it is possible that in conjunction with other enzymes added murein would be converted to its constituent parts. The second possibility is that a mixture of murein components can bind and activate the receptors responsible for ripple induction with the same efficiency as larger pieces of murein. Third, murein components might inhibit murein assembly in cells, causing them to secrete disaccharide pentapeptide that in turn would induce ripples. Further work will be required to distinguish among these possibilities.

In addition to the spontaneous appearance of ripples during fruiting body development, Reichenbach's time-lapse films record the formation of ripples by *Myxococcus* sp. feeding on *Micrococcus luteus* cells (13). Because *M. xanthus* releases murein-lytic enzymes (15) and because *M. luteus* walls are made of murein (Table 3), the ripples can be explained by the lytic release of murein or murein components from the *M. luteus* cells serving as food.

When the nutrient level was moderately high (0.5% Casitone) cells continued to grow, but could be induced by the addition of peptidoglycan to form ripples without subsequent development of fruiting bodies and myxospores. Under these conditions we have been able to observe ripples moving continuously for more than 24 h without net accumulation or depletion of cells in any macroscopic area of a petri dish 9 cm in diameter. How ripple movement is coordinated over such a large area without net cell movement is an intriguing problem. The simplest hypothesis is that cells within a ridge move forward for a half a cycle, then backward for half a cycle. At the end of one cycle the net movement would be zero. If the phase relation between adjacent ridges were correct, a visual impression of a continuously traveling wave might be obtained, much as water waves move without net displacement of water molecules. This hypothesis gives no obvious explanation for the contrary movement of ripples in adjacent tracks or of the 40° angle between the ridge line and the long cell axis. If, as an alternative scheme, cells were to move in the direction of their long axis, as gliding cells tend to do (12, 13), they would move 40° to the ridge line, thus progressing laterally in the ripple as the ripple moves forward. Lateral movement would bring cells into an adjacent track where a reversal of direction and continued progress at the same angle would eventually bring them back to their

starting point. Opposing motions in adjacent tracks would thus be responsible for maintaining an even distribution of cells, and the cross-over zones would reflect movement from one track to another. Time-lapse studies of the movement of single cells should help to distinguish these possibilities as well as other more complex cyclic trajectories.

Rippling is usually found during the early stages of development when cells are aggregating into fruiting bodies (12, 13). During development as many as 90% of the cells may lyse, and the remaining 10% may convert to myxospores (17). From developing cells, material is released which sediments at 100,000 x *g* and is capable of inducing ripples in vitro. The active material is associated with cell envelope components and may be murein released from lysing cells. There is approximately 1 mg of murein per 10^{11} cells (16). The cell density rises to 4.1×10^7 cells per cm^2 in a layer of slime that is less than 5 μm thick. The cell density inside this slime layer is therefore about 8.2×10^{10} cells per cm^3. If 90% of these cells lysed, they would release 0.74 mg of murein per cm^3. This concentration exceeds the amount of exogenous murein needed to induce ripples in submerged culture (0.5 mg/ml). Therefore the amount of murein released by autolysis during fruiting body formation is large enough to induce ripples provided it is retained within the slime layer.

A close connection between rippling and development is implied by the finding that the entire SpoC group of developmental mutants fail to ripple either in the presence or absence of murein components. The relation between rippling and development is explored further in the accompanying paper (14), where we show that the addition of murein components allows SpoC mutants to overcome their defect and to develop normally.

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