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Growth Pattern and Cell Division in *Neisseria gonorrhoeae*

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The gram-negative coccus *Neisseria gonorrhoeae* was found to grow regularly in at least two dimensions. Growth proceeded at a linear rate sequentially in each dimension. Growth in the second dimension (former width) was initiated slightly before the pole-division plane distance equalled the cell width. Penicillin treatment localized presumptive growth zones to the existing septum region. It was suggested that new growth zones were always formed perpendicular to the longitudinal axis created in the incipient daughter cells of a dividing coccus. Neither penicillin nor nalidixic acid induced filaments of *N. gonorrhoeae*. Such structures could nevertheless be formed in the rod-shaped species *Neisseria elongata*. *N. gonorrhoeae* divides by septation; however, complete septal structures with separated cytoplasms were rather infrequent. It is proposed that *N. gonorrhoeae* be regarded as a short rod which always extends parallel to the actual longitudinal axis and which never undergoes a rod-sphere-rod transition.

Studies on biogenesis of cell shape and on regulation of cell division in the gram-negative bacteria have mostly concerned the rods. Little is therefore known about these processes in the gram-negative cocci, e.g., the species of *Neisseria* (8, 21).

In a defined medium during steady-state conditions, the gram-negative rod *Escherichia coli* increases its mass by elongation in one dimension only, there being no changes in width. In slow-growing *E. coli* cells, elongation appears to proceed from the pole at which the septum was formed during the last cell division (6). After cell division, the two daughter cells grow in opposite directions. One explanation for this growth pattern would be a synthesis of envelope material from a growth zone that would duplicate at the end of each cycle (6, 25). Presumptive growth zones in *E. coli* have been localized by autoradiography (22) and may also be revealed by their specific sensitivity to penicillins (6, 11, 25). An equatorial orientation of such zones has been suggested (22).

Deviations from this growth pattern have been observed in *E. coli*. Certain *E. coli* mutants have lost their normal rod shape (15, 17). One such mutant containing the *envB* mutation changes growth direction upon cellular division, thereby giving rise to irregular shaped cocci (1). Moreover, the penicillin derivative mecillinam inhibits cell elongation and increases the cell width, thereby inducing osmotically stable cocci (12, 19). A shift from a poor to a rich medium also causes a considerable expansion of the cell width. Thus, the rod-shaped *E. coli* has the capacity to expand in both length and width. In this communication we demonstrate that *Neisseria gonorrhoeae*, unlike *E. coli* and other enterobacteria, exhibits a regular bidimensional growth pattern. The relationship between orientation of presumptive growth zones and the coccal shape will be discussed.

MATERIALS AND METHODS

Microorganisms used, growth conditions, and materials. *N. gonorrhoeae* (82409/55), colony type 4, was obtained from Alice Reyn, Copenhagen, Denmark. *Neisseria elongata* was kindly supplied by Kjell Bøvre, Tromsø, Norway. The bacteria was stored at −60°C in GC medium base (GCMB) (Difco) from which agar was omitted, plus supplement B (Difco) and 20% glycerol.

Cultivation on solid medium was performed on GCMB plus supplement B at 36°C in a CO_2 incubator (6% CO_2). The liquid medium used was GCMB plus supplement B, except agar was omitted. Growth was performed in the presence of 10% CO_2 in air or in medium containing 10 mM bicarbonate (28). Growth was followed in a Klett-Summerson photometer (red filter) or in a Zeiss spectrophotometer at a wavelength of 450 nm.

Penicillin G was purchased from AB Kabi, Stockholm, Sweden. Nalidixic acid was from Winthrop Laboratories, Newcastle upon Tyne, England. Mecillinam was a gift from AB Løvens, Ballerup, Denmark. [^{3}H]adenine (22 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, England.

Phase-contrast microscopy. GCMB was dissolved

during shaking and heating to about 80°C. At 50°C supplement B was added, and the melted agar solution was gassed for 1 min with 10% CO_2 in air. One milliliter was poured onto a glass slide and allowed to harden for about 5 min in a petri dish. Small holes, arranged in a circle, were made in the agar, and 25 μl of a suspension of bacteria (about 10^6) in the logarithmic growth phase was placed within this circle on the agar surface. The small holes were filled with CO_2, and the inoculated area and the circle of holes were immediately covered with a cover slip. Agar pieces outside the cover slip were cut away, and it was sealed with vaseline. The microculture was kept at 37°C by use of a heated stage (Zeiss), and the growth of individual cells was followed in a Zeiss phase-contrast microscope with camera equipment. The film negatives were projected on a screen at a fixed distance, and cell dimensions were determined by direct measurements.

Scanning electron microscopy. An 0.1-ml amount (about 10^7 bacteria) of a cell suspension in the logarithmic growth phase was spread on GCMB plates with or without the addition of drugs. The plates were incubated for various times. The processing for scanning electron microscopy was performed according to Elmros et al. (7).

Transmission electron microscopy. The cells to be studied were harvested by centrifugation (2,000 $\times$ *g*) for 10 min at 4°C. Fixation and preparation for electron microscopy was carried out as previously described (18).

Determination of DNA and RNA synthesis. Cells were pregrown in GCMB containing 0.01 M $NaHCO_3$ for several generations. Incorporation of [^{3}H]adenine (15 μCi/ml) was followed. Incorporation into ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) was followed in samples (200 μl) precipitated in 5 ml of ice-cold 10% trichloroacetic acid which were allowed to precipitate at 0°C for at least 30 min (trichloroacetic acid-precipitable material). Samples for DNA determination were hydrolyzed overnight in 0.5 ml of 0.5 M NaOH and afterwards precipitated with trichloroacetic acid as described above (alkali-stable material). Each sample was then filtered through a Whatman glass filter (25 mm) and washed five times with ice-cold 5% trichloroacetic acid containing adenine (10 μg/ml), once with boiling water, and once with acetone. The filters were dried and counted in a liquid scintillation counter (Nuclear-Chicago Mark II).

RESULTS

Growth pattern of *N. gonorrhoeae* type 4. Growth of individual cells was followed during incubation on agar slides. The cell shown in Fig. 1 expands perpendicular to the division plane. However, growth in this dimension ceased, and the developing daughter cells initiate growth parallel to the existing septum. As a consequence, consecutive division planes will be formed at right angles to each other, giving rise to tetrads of cells. This bidimensional

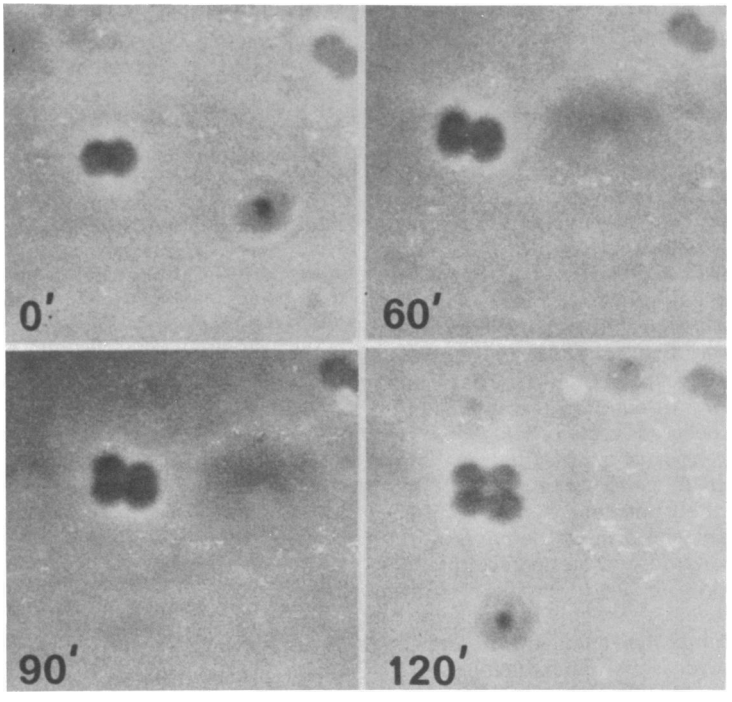

FIG. 1. *Growth of "type 4 gonococcal cells." Note the perpendicular orientation of two consecutive division planes.* ×6,550.

growth pattern was observed in all gonococcal cells examined. It was also found in other *Neisseria* species such as *N. meningitidis* and *N. pharyngitis*.

The growth in two dimensions of six gonococci was followed on agar slides (Fig. 2). The following observations were made. (i) Expansion of individual gonococci proceeded in only one dimension throughout virtually the entire cell cycle. However, expansion in two dimen-

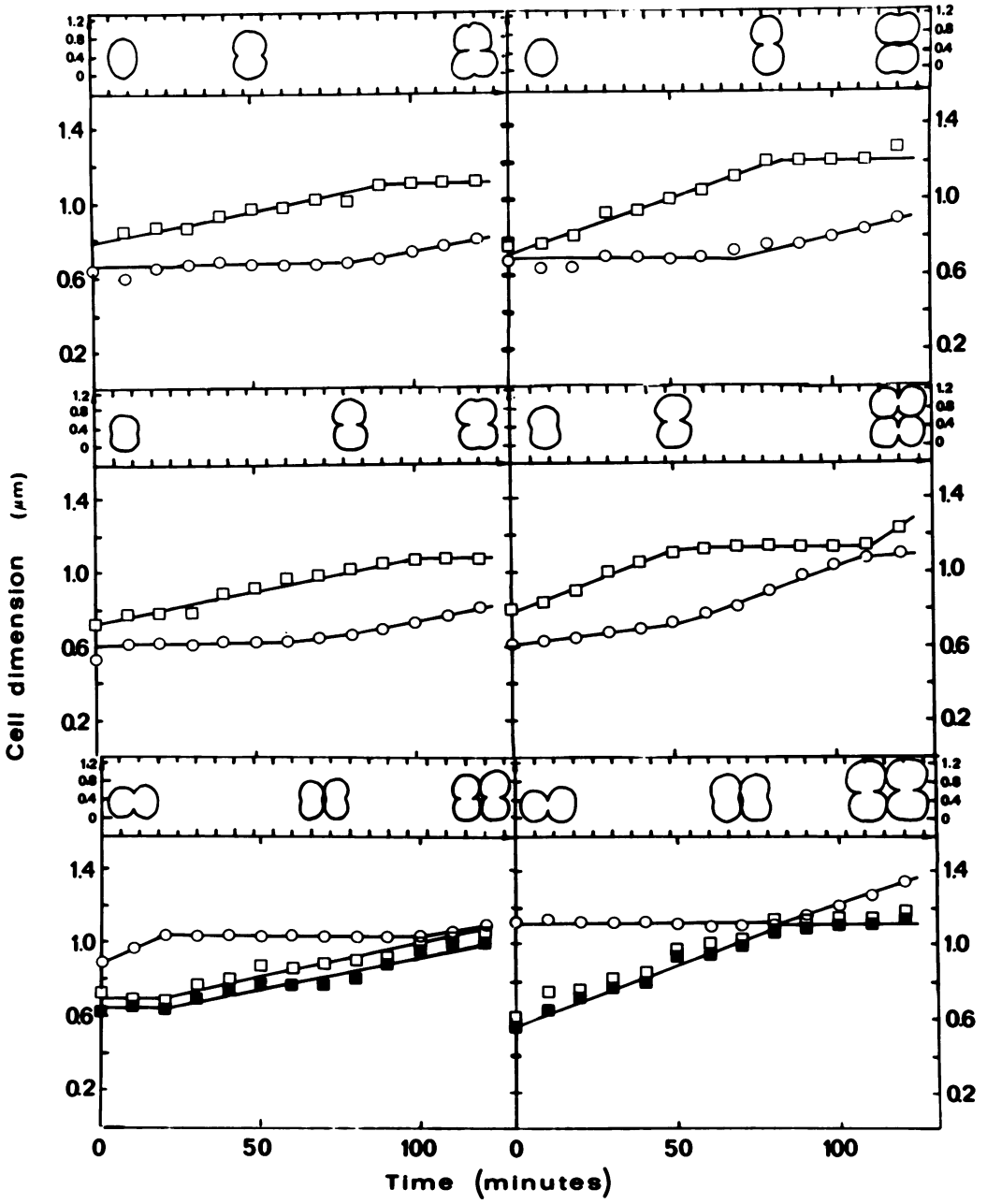

FIG. 2. *Bidimensional growth of six independent type 4 gonococci. The development of the respective gonococcal cell is drawn above each diagram. The vertical (squares) and horizontal extension (circles) was followed. The two gonococci at the bottom of the figure were initially in a late stage of septation. The vertical extension of each of the developing daughter cells was therefore followed (closed and open squares).*

sions was observed in some cells at the end of the division period. (ii) The rate of extension in each dimension appeared to be linear. (iii) Growth in the second dimension (former width) was initiated slightly before the distance from one pole to the division plane equalled the cell width.

Presumptive growth zones in *N. gonorrhoeae*. At present there are no direct methods for visualization of envelope growth zones in *N.*

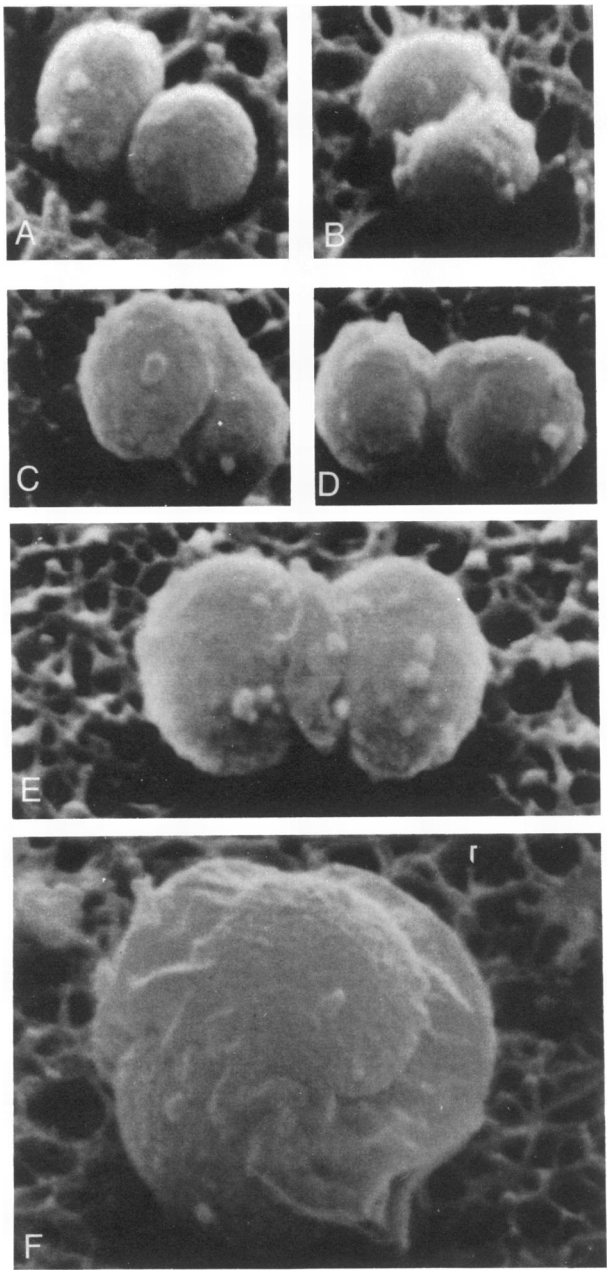

FIG. 3. *Scanning electron micrographs of type 4 gonococcal cells incubated on GMCB agar for 60 min (A and B, controls) and incubated on GCMB agar supplemented with 1 μg of penicillin G per ml of agar medium for 60 (C), 90 (D), 120 (E), and 240 min (F). Note the occurrence of a protruding area in the invagination region of the penicillin-treated gonococci (C–F). In both controls and penicillin-treated gonococci spherical bodies of unknown nature are seen on the surface.* ×24,000.

gonorrhoeae. We have chosen to study the morphological effect on *N. gonorrhoeae* of penicillin G. A series of scanning electron microscopic observations were made on *N. gonorrhoeae* type 4 cells growing on solid medium in the presence of penicillin G (1 μg/ml). The effect of 1 to 2 h of penicillin treatment was restricted to the septal region, which showed a protruding area (Fig. 3C, D, and E). Even after 4 h of penicillin exposure, the polar caps appeared conserved, whereas the septal region was heavily distorted (Fig. 3F). Blebs of an unknown nature were observed both in the control and in the drug-exposed cells (Fig. 3). In 33 cells both septum region and poles were visible. In all these cells penicillin-induced weakenings showed an equatorial orientation. In the transmission electron microscope similar weakenings of the septal region were observed after penicillin treatment. In rare cases, cells were found that contained two protrusions perpendicular to each other (Fig. 4). Bulges with clear connection to the cell were not found outside the existing septum region.

Septal structures in *N. gonorrhoeae* type 4. *N. gonorrhoeae* divides by septation and not by constriction (Fig. 5). Cell division is initiated by an ingrowth of cytoplasmic membrane enclosing a fold of peptidoglycan (Fig. 6A). This gives rise to partial or complete septal structures. The septal peptidoglycan was often visualized as two distinct layers (Fig. 6A and B). In a few cells (3 out of 25 with complete septa; Fig. 6C), the cytoplasmic membrane together with one fold of peptidoglycan formed an ingrowth perpendicular to the septum.

It has been suggested that residual activity of autolysins may derange native septal structures (2, 3). One argument for the presence of both partial and complete septa in vivo was the observation that mecillinam added to dividing gonococci either induced one spheroplast (Fig. 7A) or caused the formation of two individual spheroplasts, one lysing before the other (Fig. 7B and C). In the former case a communicating cytoplasm is likely present, whereas a complete septum must exist in the latter cells examined.

Comparative effects of nalidixic acid and penicillin G on *N. gonorrhoeae* and *N. elongata*. At present no gonococcal mutants are available showing a unidimensional growth pattern. However, a rod-forming species of *Neisseriaceae* has been isolated, *N. elongata* (4, 5). On agar slides this species grows in one dimension only. Moreover, unlike *N. gonorrhoeae*, *N. elongata* maintains the same orientation of the longitudinal axis throughout several generations.

Nalidixic acid and penicillin G are drugs known to induce filamentous growth of gram-

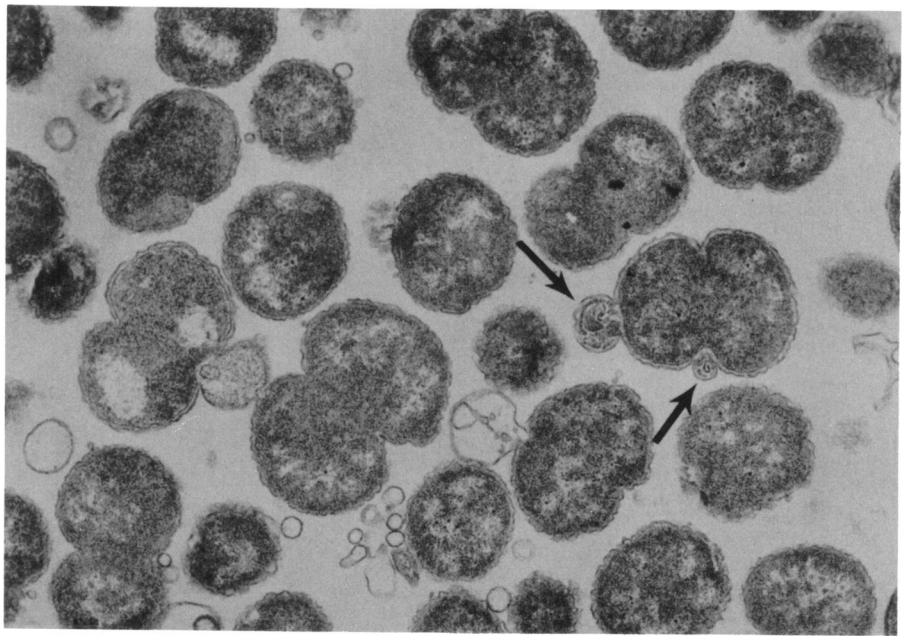

FIG. 4. *Transmission electron micrograph of gonococcal cells grown for 2 h in liquid medium (GCMB with agar omitted) supplemented with penicillin G (1 μg/ml). Note the cell with two protrusions located perpendicular to each other (arrows). ×17,400.*

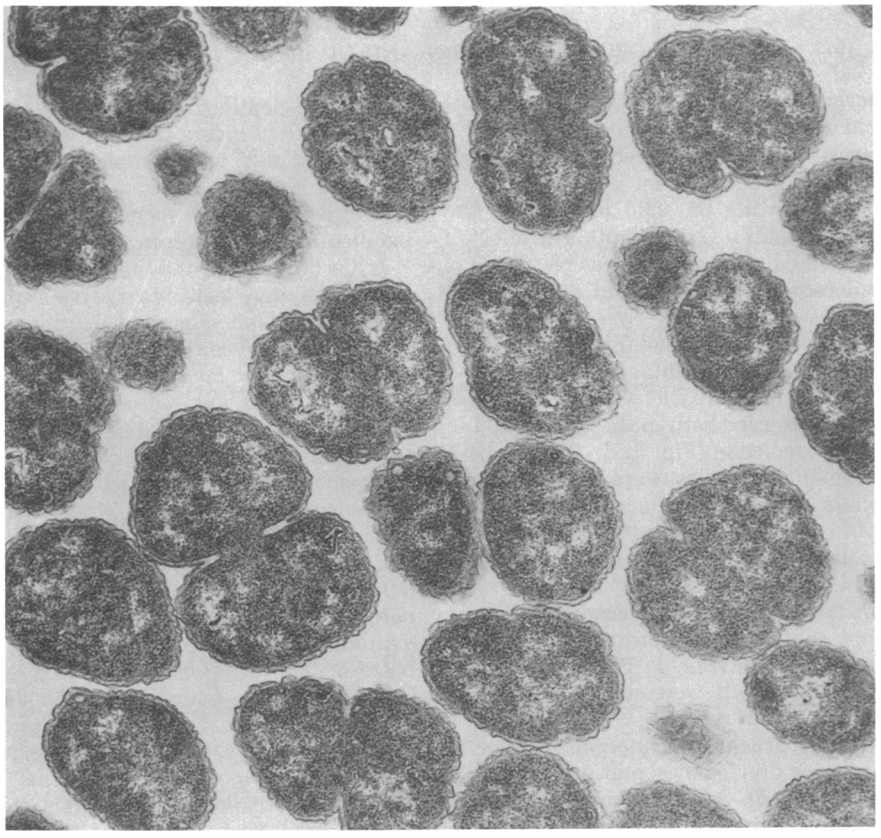

FIG. 5. *Survey transmission electron micrograph of type 4 gonococcal cells. Note that most cells during division exhibit only partially developed septa. ×27,100.*

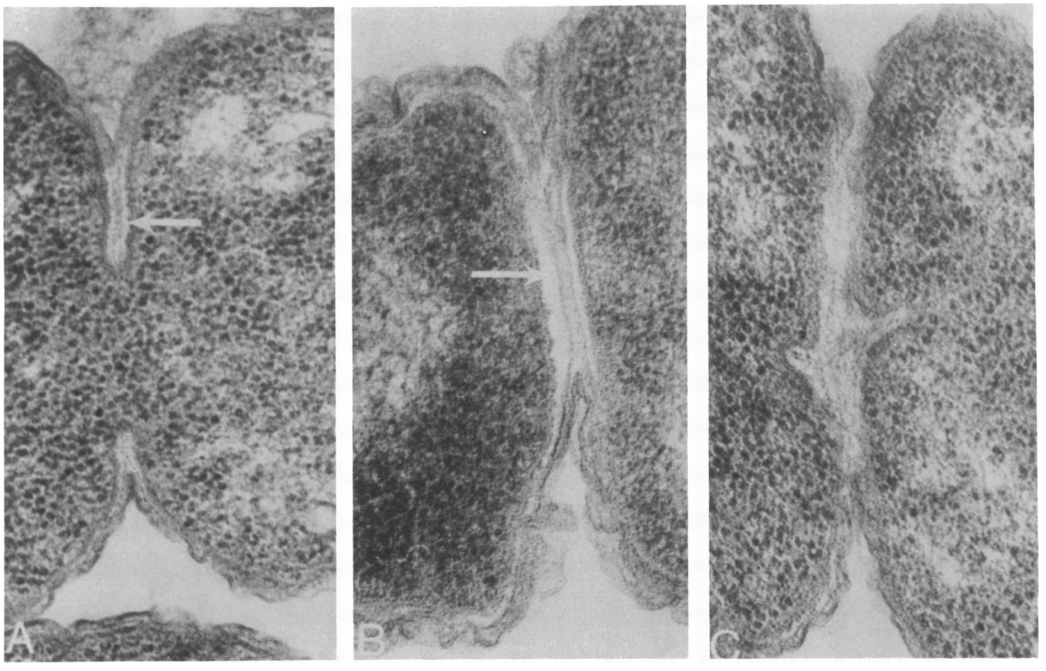

FIG. 6. *Different stages in septum development. Two separate septal peptidoglycan layers may be discovered (arrows, A and B). Note in (C) that the cytoplasmic membrane together with the peptidoglycan layer form an ingrowth perpendicular to the complete septum. (A and C) ×79,000; (B) ×118,000.*

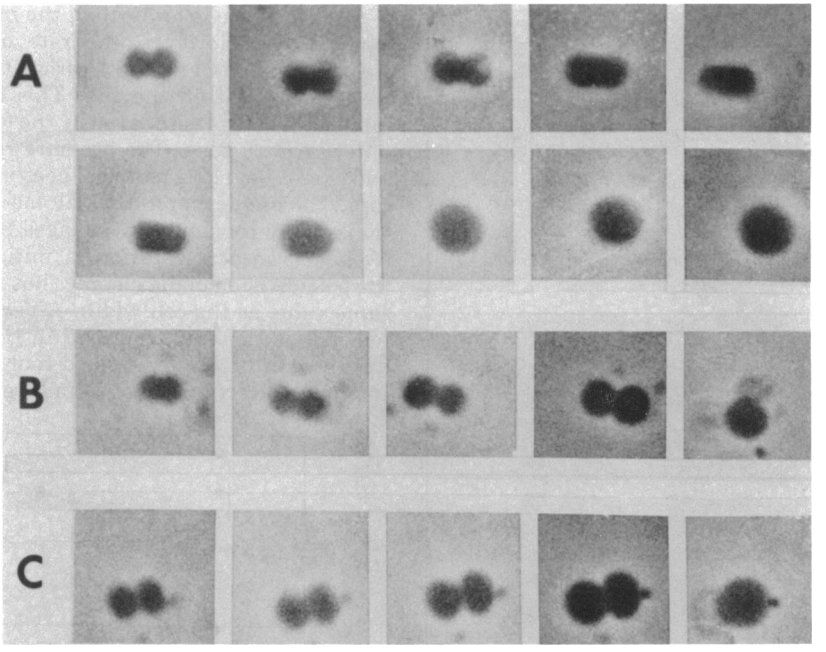

FIG. 7. *Mecillinam-induced changes of three dividing type 4 gonococcal cells grown on GCMB supplemented with mecillinam (1 μg/ml). Photographs in each series were taken at intervals within a time span of 2 h. Note the induction of either one (A) or two (B and C) spheroplasts. ×6,900.*

negative rods (9, 25). When *N. elongata* was treated with nalidixic acid (5 μg/ml), DNA synthesis was inhibited, whereas RNA synthesis continued (Fig. 8). Due to agglutination, the mass increase was difficult to follow. However, on agar slides, short, nonseptated filaments could be visualized after nalidixic acid treatment. In contrast, in *N. gonorrhoeae*, nalidixic acid (5 μg/ml) caused an almost instantaneous inhibition of mass increase and of RNA as well as DNA synthesis (Fig. 9). The drug also inhibited completion of cell separation. Likewise, penicillin G (1 μg/ml) induced filaments of *N. elongata* and round, large cells of *N. gonorrhoeae*.

DISCUSSION

The shape and dimension of a bacterium reflect the processes by which the cell envelope or shape determining structures grow. In rod-shaped bacteria evidence has been provided for both a dispersed and a localized synthesis of cell envelope (6, 11, 13, 16, 22–24, 26). In *E. coli*, at least certain envelope components, notably the peptidoglycan layer, seem to be layed down in annular zones (22, 26). In the gram-positive coccus *Streptococcus faecalis*, it seems convincing that peripheral wall extension proceeds from specific growth zones. After cessation in length extension from a particular growth site it continues to develop a septum. The growth zone is thereafter inactivated, and new zones are formed de novo at the junction of old and new walls (10, 27). In *N. gonorrhoeae*, penicillin-induced bulges of the envelope are restricted to the septal region. This may indicate that this region represents the region of active growth.

It has been suggested that bacteria showing a unidimensional growth pattern extend linearly, with doublings in rate in relation to the formation of new growth zones (14, 29). However, experimentally it has been very difficult to distinguish this growth pattern from that of a cell extending exponentially from a large number of sites. In *N. gonorrhoeae* exhibiting a bidimensional growth pattern, growth in each dimension apparently proceeds linearly. This again suggests a localized formation of shape-determining structures.

N. gonorrhoeae, like most gram-negative bacteria, divide by septation. The constrictions observed may, as pointed out by Burdett and Murray (2, 3), be artifacts due to partial autolysis during the preparation procedure. Complete septa were visualized rather infrequently. They consisted of two, often separate layers of peptidoglycan and were delimited by the cytoplasmic membrane of each incipient daughter cell. At times cytoplasmic ingrowths perpendicular

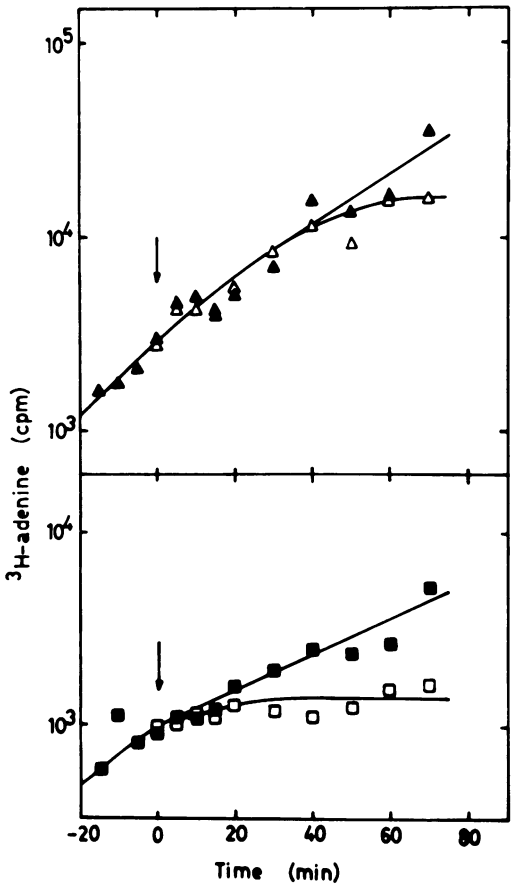

FIG. 8. *Effect of nalidixic acid (NAL) on the incorporation of [^{3}H]adenine into trichloroacetic acid-precipitable (DNA and RNA) and alkali-stable (mostly DNA) material in N. elongata. [^{3}H]adenine (15 μCi/ml) was added to growing cells of N. elongata at −20 min. At zero time the culture was divided into two parts; NAL acid (5 μg/ml) was added to one part and the other served as a control. Due to cell aggregation growth measured as absorbance at 450 nm was not possible to follow. Each value given was the mean of four different samples. Symbols: ▲, control, trichloroacetic acid-precipitable material; △, +NAL, trichloroacetic acid-precipitable material; ■, control, alkali-stable material; □, +NAL, alkali-stable material.*

to the septum were observed, suggesting that new divisions may be initiated before the outer membrane has completed the previous one. Similar structures have been observed in a sphere-like mutant of *E. coli* containing the *envB* mutation (1).

Pritchard (20) has presented a simple model for cell shape and division which involves a self-regulating system in which a linearly extending surface area encloses an exponentially expanding cell mass. In the newborn cell the rate of mass increase is lower than the rate of envelope growth. However, the rate of mass increase augments and the hydrostatic pressure is increased within the cell. This increase in internal pressure is relieved by the formation of a new growth zone. The pressure rapidly falls and excess envelope material goes into the septum. The oscillation in the volume-to-mass ratio is finely regulated by changes in width. In *N. gonorrhoeae*, septation is initiated before the pole-division plane distance has reached the same value as the cell width. A new longitudinal axis is thereby generated in the incipient daughter cells. It is suggested that the change in growth direction observed in *N. gonorrhoeae*

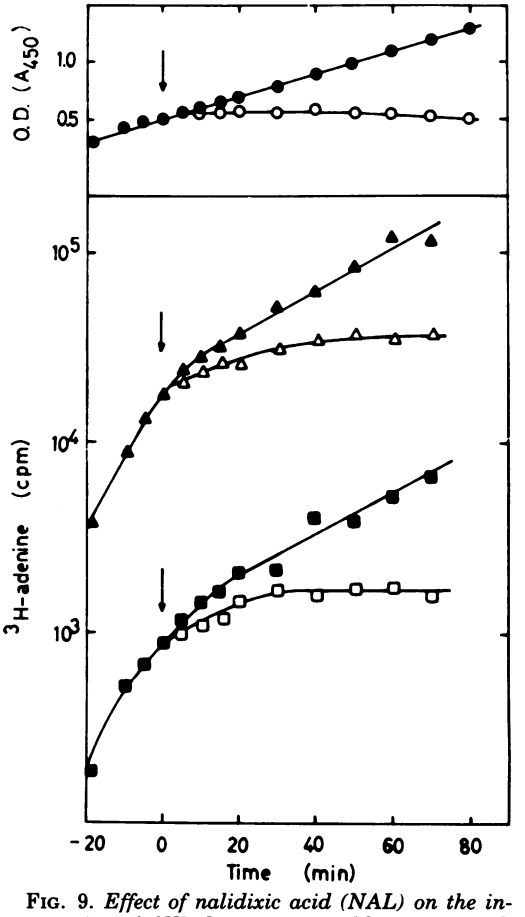

FIG. 9. *Effect of nalidixic acid (NAL) on the incorporation of [^{3}H]adenine into trichloroacetic acid-precipitable (DNA and RNA) and alkali-stable (mostly DNA) material in N. gonorrhoeae (type 4). The experimental conditions were as in Fig. 8. Symbols: ●, control, optical density; ○, +NAL, optical density; ▲, control, trichloroacetic acid-precipitable material; △, +NAL, trichloroacetic acid-precipitable material; ■, control, alkali-stable material; □, +NAL, alkali-stable material.*

reflects a way to avoid a rod-sphere-rod transition, which would occur if growth was unidimensional. Such a transition should cause large oscillations in the volume-to-mass ratio and perhaps too large variations in the internal hydrostatic pressure. If as suggested by Pritchard (20) resistance to extend in length is lower than resistance to expand in width, then bidimensional growth should exist in short rods where longitudinal axes of parent and incipient daughter cells are perpendicular to each other.

Penicillin G and nalidixic acid are known to induce filaments of rod-shaped bacteria (9, 25). This was also true for the rod-shaped *N. elongata*. In contrast, filaments of *N. gonorrhoeae* could not be induced with either of these agents. Penicillin induced large spheres, whereas nalidixic acid rapidly inhibited gonococcal growth together with RNA and DNA synthesis. The latter drug, at the same concentration, induced short filaments and selectively inhibited DNA synthesis in *N. elongata*. Nalidixic acid has been suggested to affect cell division by inhibiting formation of new growth zones (30). In *E. coli* this leads to a constant rate of envelope extension and filamentation. It seems likely that in *N. gonorrhoeae* growth in the presence of nalidixic acid is inhibited when the pole-division plane distance has reached the same value as the cell width; i.e., a sphere-rod transition is not possible. The loss of a longitudinal axis may be a relevant factor for this inhibition (1).

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