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Bacterial Cell Shape Regulation: Testing of Additional Predictions Unique to the Two-Competing-Sites Model for Peptidoglycan Assembly and Isolation of Conditional Rod-Shaped Mutants from Some Wild-Type Cocci

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The two-competing-sites model for peptidoglycan assembly for bacterial cell shape regulation suggests that in rods, bacterial cell shape depends on the balance between two reactions (sites), one responsible for lateral wall elongation and the other responsible for septum formation. The two reactions compete with each other so that no lateral wall can be formed during septum formation and vice versa. When the site for lateral wall elongation overcomes that for septum formation, long rods or filaments are formed and cell division may be blocked. When the reaction leading to septum formation is hyperactive compared with the other, coccobacilli or cocci are formed. Other bacteria carry only one site for peptidoglycan assembly and can grow only as cocci. The two-competing-sites model predicts that two different types of cocci exist (among both morphology mutants and wild-type strains); one carries only the site for septum formation, whereas the other also carries the site for lateral wall elongation, the former site predominating over the latter. As a consequence of the inhibition (by antibiotics or by mutations) of septum formation in wild-type cocci of various species and in coccoid morphology mutants, some cocci are expected to undergo transition to rod shape and others are not. We have evaluated these predictions and show that they are in agreement. In fact, we found that among wild-type cocci belonging to 13 species, those of 6 species formed rods, whereas the remaining organisms maintained their coccal shape when septa were inhibited by antibiotics. Some coccoid morphology mutants of rod-shaped bacteria underwent coccus-to-rod transition after septum inhibition by antibiotics, whereas others maintained their coccal shape. When a mutation that causes septum inhibition was expressed in a morphology mutant of *Klebsiella pneumoniae* grown as a coccus, transition to rod shape was observed. A total of 914 mutants unable to form colonies at 42°C were isolated from the coccoid species mentioned above. Between 75 and 95% of the mutants isolated from the species that formed rods when septum formation was inhibited by antibiotics but none of those isolated from the others underwent coccus-to-rod transition upon incubation at the nonpermissive temperature.

In previous papers, we proposed a model for shape regulation in bacteria and presented a large body of experimental data in support of it (33, 34, 37). In this study, we have extensively tested predictions concerning the effects on cell shape of septum inhibition by antibiotics and mutations in various wild-type and mutant coccoid bacteria (see predictions 6 and 7 of Table 1, which have previously been evaluated only preliminarily). We show that all predictions are fulfilled and demonstrate that some coccoid species but not others undergo transition to rod shape when septa are specifically blocked by antibiotics and that mutants undergoing coccus-to-rod transition can be isolated in some coccoid species but not in others.

The two-competing-sites (TCS) model for peptidoglycan assembly (TCS) for bacterial shape regulation (Fig. 1) mentioned above suggests that shape determination and maintenance in bacterial rods depends on the activity of two biochemical reactions (sites) which occur in the terminal stages of peptidoglycan synthesis; one site is responsible for lateral wall elongation, and the other is responsible for septum formation (34, 37). The two sites compete with each other in such a way that the lateral wall is not extended during septum formation and vice versa (33). The actual shape of the bacteria is thus determined by the balance between the two competing reactions, correct balance leading to normal rods; abnormal prevalence of the site for lateral wall elongation leads to long rods or filaments, whereas prevalence of the site for septum formation leads to formation of coccobacilli or cocci (10, 15, 33–35, 37). Other bacteria carry only one site for peptidoglycan assembly; such strains can form only septa and can grow only as cocci.

This model is based on the suggestion by Schwarz et al. that in rod-shaped bacteria two separate functions may exist, one responsible for lateral wall elongation and the other responsible for septum formation (38). This suggestion was taken up by Higgins and Shockman (20), who proposed that in contrast to rods, cocci expand their surfaces only through cross wall formation and that rods must carry additional genetic information for the production of lateral walls. Others (13, 18, 23, 28, 39, 41–44, 46) have supported these proposals.

The TCS model takes the proposal by Schwarz et al. (38) and adds to it the suggestion that the two reactions compete with each other so that in undisturbed bacteria they are mutually exclusive (and therefore alternate during the cell cycle) and that bacterial cell shape and (to a certain extent)

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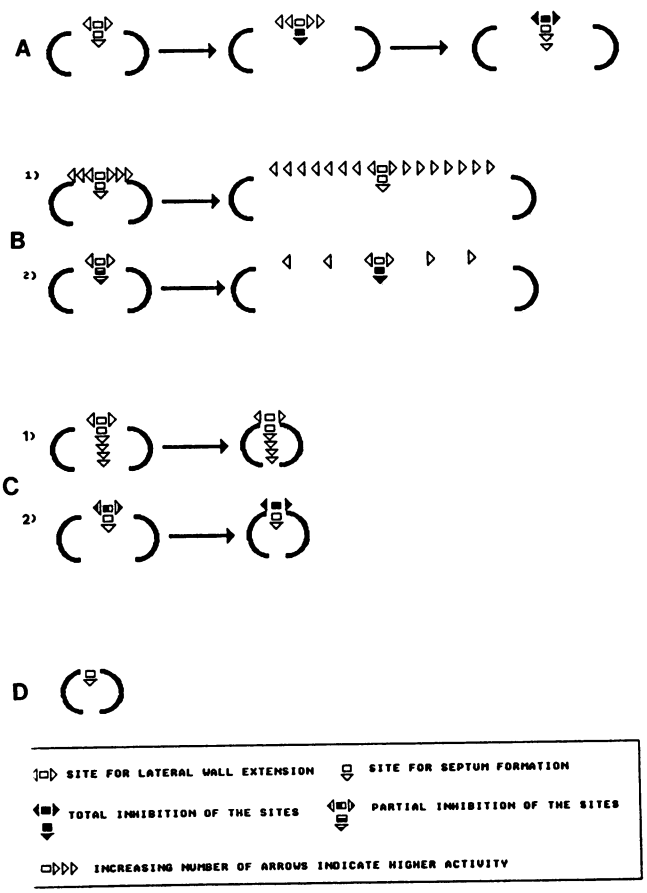

FIG. 1. Schematic depiction of the TCS model for shape regulation in bacteria. In this model, some bacteria carry two sites for peptidoglycan assembly, which compete with each other. In these bacteria (A), normal rod shape depends on the regulated alternation of the activities of the sites. (B) Relative hyperactivity of the site for lateral wall elongation leads to formation of long rods or filaments (and inhibition of cell division); such relative hyperactivity can be due either to increased activity of the site (B1) or to decreased activity or inhibition of the site for septum formation (B2). (C) Relative hyperactivity of the site for septum formation leads to formation of coccobacilli or cocci; such relative hyperactivity can be due either to a greater efficiency of the site for septum formation (C1) or to a reduced activity or inhibition of the site for lateral wall extension (C2). (D) Other bacteria carry only one site for peptidoglycan assembly and can only grow as cocci.

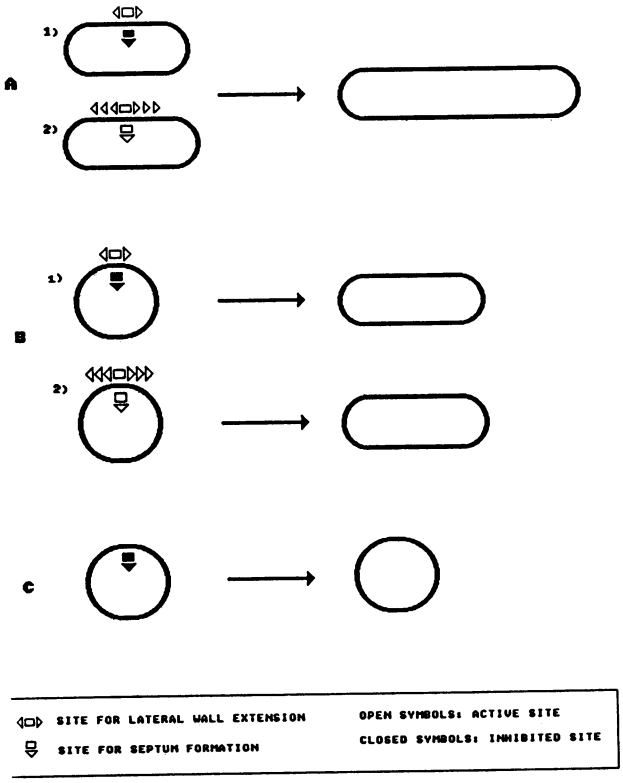

FIG. 2. Some predictions allowed by the TCS model for shape regulation in bacteria. These predictions refer only to the effects of interferences of various nature on cell shape and cell division of coccoid and rod-shaped bacteria. (A) When in rod-shaped bacteria the septum is inhibited or impaired by mutations (or increased gene dosage) or antibiotics (or other chemicals) (A1), or when the activity of the lateral wall elongation site is enhanced by mutations (or increased gene dosage) or by antibiotics (or other chemicals) (A2), filaments are formed and cell division is inhibited. (B) When in coccoid bacteria that carry two sites for peptidoglycan assembly septum formation is inhibited by mutations (or increased gene dosage) or by antibiotics (or other chemicals) (B1), or when the lateral wall elongation site is enhanced by mutations (or increased gene dosage) or by antibiotics (or other chemicals) (B2), cocci are converted in rods. (C) When in coccoid bacteria that carry only one site for peptidoglycan assembly septum formation is inhibited by mutations (or increased gene dosage) or by antibiotics (or other chemicals), cell surface extension is blocked and coccal shape is maintained.

division are determined by the balance of these competing reactions. This concept, peculiar and exclusive to the TCS model, is not obvious, nor is it a minor modification of previous proposals (38), since it confers on the TCS model a number of unique properties. Such a concept endows the TCS model with a peculiar kind of dynamic property whereby bacterial cell shape can be modified in all possible ways (from rod to filament or coccus and vice versa) simply by interfering with the balance of the two competing reactions; a given shape change can be caused either by inactivation or weakening of a site or by enhancement of the activity of the reaction responsible for the competing function (34, 37). Similarly, a round configuration of wild-type cocci is not necessarily dependent on the fact that such cells carry only the site for septum formation; another type of wild-type cocci may also exist. Like rods, such cocci carry two sites for peptidoglycan assembly, but in these cells the reaction leading to septum formation is stronger and prevents the lateral wall from being formed (34, 37). In addition, in the TCS model, cell division may depend on events connected with bacterial morphogenesis, since the reaction leading to septum formation has to compete with that responsible for lateral wall elongation and may be abnormally overcome by it, thus leading to inhibition of cell division. Therefore, formation of filaments does not necessarily imply damage in the site for septum formation, but hyperactivity of the lateral wall elongation reaction may also have a similar effect (34, 37) (Fig. 2).

The uniqueness of the TCS model is further demonstrated by certain predictions it affords, which are not feasible with other models. These predictions are summarized in Table 1 and Fig. 2.

(This work was presented in part at the FEMS Symposium on the Murein Sacculus of Bacterial Cell Walls, Architecture and Growth, Berlin, 1983.)

TABLE 1. Major predictions of the TCS model for bacterial shape regulation

Prediction	Fulfilled?	Reference
1. The transition from coccal to rod shape implies activity of an additional site for peptidoglycan assembly and will be accompanied by increased peptidoglycan synthesis and vice versa in the reverse shape transition.	Yes	37
2. In cocci that grow synchronously, peptidoglycan is made only for forming septa, and this polymer will be synthesized only when septum formation takes place.	Yes	13, 37
3. Since bacterial rods carry two different reactions for forming peptidoglycan, some β-lactams will be more active on one or the other of the two reactions.	Yes	37, 41
4. Cocci carry only the site for septum formation in an active form. They will be resistant to antibiotics that specifically act on the site for lateral wall elongation (such as mecillinam).	Yes	2, 33, 34
5. In some mutants that form filaments, inability to form septa depends on inactivation of the site for septum formation, whereas in others it may depend on the relative hyperactivity of the site for lateral wall elongation. The latter mutants, but not the former, will be made to divide by an antibiotic that inhibits lateral wall elongation or by an additional mutation that restores balance between the two competing reactions.	Yes	10
6. In some coccoid morphology mutants, coccal shape should be due to inactivation of the site for lateral wall formation; in others, it should be due to relative hyperactivity of the site for septum formation. In the latter mutants, but not in the former, antibiotics or mutations that restore the balance between the two sites will cause cocci to assume the rod shape (see also Fig. 2).	Only preliminary	This study; 34
7. Some wild-type cocci carry only the site for septum formation, whereas others also carry the site for lateral wall extension, which is overcome by the other site and is not expressed under ordinary conditions. Antibiotics or mutations that impair the site for septum formation (or stimulate the site for lateral wall extension) will not affect the shape of the former cocci but will make the latter undergo coccus-to-rod transition (see also Fig. 2).	Only preliminary	This study; 34
8. Mutations conferring coccal shape to ordinary rod bacteria can suppress the filamentation phenotype in mutants unable to form septa, making them capable of dividing and vice versa.	Yes	5
9. Increased dosage of genes involved in lateral wall elongation will allow the lateral wall elongation site to constantly overcome the septum formation site, leading to formation of filaments and inhibition of cell division.	Yes	49
10. Antibiotics or other substances stimulating activity of the lateral wall elongating site will have similar effects.	Yes(?)	21, 47, 48
11. Increased dosage of genes involved in septum formation will allow the site responsible for this reaction to constantly overcome the lateral wall elongation site, causing formation of coccobacilli or cocci.	NT[a]	
12. Similar effects will be caused by antibiotics or other substances stimulating activity of the site for septum formation.	NT	

[a] NT, Not tested.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used are shown in Table 2. Brain heart infusion broth (Difco Laboratories, Detroit, Mich.) was used for all but the following strains: *Klebsiella pneumoniae* MIR M7 (peptone-lactose medium buffered at pH 7 or 5.8 [33]) and *Neisseria* strains (brain heart infusion broth supplemented with 10% calf serum). All cultures were incubated at 37°C with shaking.

Optical density of the cultures was measured with a Beckman model DU 6 spectrophotometer at 640-nm wavelength. Cell number was estimated with a Coulter Counter model ZBI equipped with a 30-μm capillary as previously described (11).

Susceptibility test and morphology studies. The minimal concentration of antibiotic inhibiting cell number increase was determined by counting the cell number of the cultures with the Coulter Counter after 3 h of incubation in the presence of different concentrations of antibiotic. Samples for microscopic observation were prepared as previously described (10). Photomicrographs were obtained with a Leitz Orthoplan microscope equipped with an automatic camera.

Isolation of cell growth thermosensitive mutants. A 10-ml sample of exponential-phase cultures of wild-type strains was added with 0.3 ml of ethyl methanesulfonate and incubated at 32°C for 60 min. Cells were washed twice and incubated overnight in brain heart infusion broth at 32°C. Mutagenized cultures were diluted, plated on brain heart agar and incubated at 32 and 42°C. Plates containing 100 to 300 colonies were replicated and incubated at 32 and 42°C. Clones that formed colonies at 32 but not 42°C were considered to be thermosensitive cell growth mutants.

A certain percentage of these mutants (see Table 6) increased their number at 42°C for only 15 min and then stopped dividing. At this temperature, cells elongated and then formed septa, but new cells could not separate or separated only partially. Other mutants (a minor percentage; see Table 6) divided only for 1 h or less and then stopped dividing; they always maintained their coccal shape at 42°C. In any case, macromolecular synthesis continued only during cell elongation or when cells were dividing; when cells stopped dividing or elongation was completed, no more DNA or proteins were snythetized.

Analysis of DNA synthesis. Exponentially growing cultures were labeled with 2 μCi of [^{3}H]thymidine. The trichloroacetic acid-precipitable isotope incorporation was determined by placing 0.5-ml samples in 4.5 ml of 10% trichloroacetic acid on ice for 30 min. Samples were then collected on glass microfiber filters (GF/C; Whatman, Inc., Clifton, N.J.) and washed, and the radioactivity was counted as described previously (32).

Assay for PBPs. Penicillin-binding proteins (PBPs) of *Neisseria catarrhalis* were detected as described by Dougherty et al. (14). Analysis of PBPs in streptococci was performed as previously described. Briefly, exponentially growing cells

TABLE 2. Bacterial strains used

Species	Strain(s)	Genotype and properties	Source	Reference
Proteus mirabilis	VR1	Wild type	Our collection	
Acinetobacter calcoaceticus	VR1, VR2	Wild type	Our collection	
Escherichia coli	SP6	*pbpA* PBP2$^-$, coccoid at 32 and 42°C	B. G. Spratt	46
	SP45	*pbpA* PBP2$^-$(Ts), coccoid at 42°C	B. G. Spratt	46
	SP52	*rodA*(Ts), coccoid at 42°C	B. G. Spratt	46
	envB	*envB*, coccoid at 32 and 42°C	S. Normark	50
	KJB 20	*pbpA*(Ts) *ftsZ*(Ts)	K. J. Begg	4
Klebsiella pneumoniae	MIR M7	Coccoid at pH 7, rod at pH 5.8, temperature sensitive for cell division	G. Satta	33
Neisseria gonorrhoeae	NG1, NG2, NG3	Wild type	Our collection	
N. catarrhalis	NC1, NC2, NC3	Wild type	Our collection	
Staphylococcus aureus	VA1, VA2, VA3, VA4, VA5	Wild type	Our collection	
Staphylococcus simulans	VA1, VA2, VA3, VA4	Wild type	Our collection	
Staphylococcus intermedius	VA1, VA2, VA3, VA4	Wild type	Our collection	
Streptococcus lactis	VR1, VR2	Wild type	Our collection	
Streptococcus disgalactiae	VR1, VR2, VR3	Wild type	Our collection	
Streptococcus bovis	VR1, VR2, VR2	Wild type	Our collection	
Streptococcus agalactiae	VR1, VR2	Wild type	Our collection	
Streptococcus faecium	VR1, VR2	Wild type	Our collection	
	ATCC 9790	Wild type	G. D. Shockman	
Streptococcus sanguis	VR1	Wild type	Our collection	
Streptococcus sanguis	Challis	Wild type	F. L. Macrina	24
Streptococcus lactis	ML3	Temperature sensitive for cell growth	This study	
Streptococcus disgalactiae	MD5	Temperature sensitive for cell growth	This study	
Streptococcus agalactiae	MA37	Temperature sensitive for cell growth, rod at 42°C	This study	
Streptococcus bovis	MB71	Temperature sensitive for cell growth, rod at 42°C	This study	
Streptococcus faecium	NON-11	Temperature sensitive for cell growth, rod at 42°C	This study	
Streptococcus sanguis	MS80	Temperature sensitive for cell growth, rod at 42°C	This study	
Staphylococcus aureus	ts-6	Temperature sensitive for cell growth	This study	
Staphylococcus simulans	ts-12	Temperature sensitive for cell growth	This study	

were harvested by centrifugation, washed with 10 mM potassium phosphate buffer (pH 7), and broken with a Standsted cell disruptor (Stansted Fluid Ltd., Stansted, England) operating at 35,000 lb/in^2. Membranes were purified by differential centrifugation and resuspended at a protein concentration of 40 mg/ml. A 50-μl sample of membrane suspension was incubated with [^{14}C]benzylpenicillin (final concentration, 100 μM) at 37°C for 60 min. After membrane solubilization, proteins were separated by sodium dodecyl sulfate-polyacrylamide slab gels and PBPs were visualized by fluorography (16).

Antibiotics and other chemicals. Nalidixic acid and mecillinam were from Sigma Chemical Co., St. Louis, Mo. Mitomycin C was from Serva Feinbiochemica, Heidelberg, Federal Republic of Germany. Penicillin G and aztreonam were from E. R. Squibb & Sons, Princeton, N.J. [^{3}H] thymidine (specific activity, 20 Ci/mmol) and [^{14}C]benzylpenicillin (specific activity, 60 mCi/mmol) were from The Radiochemical Centre, Amersham, England. All reagents were of the highest purity available.

RESULTS

Effects of septum inhibition on cell shape of wild-type cocci. The analysis involved several strains of each of nine gram-positive (three staphylococcal and six streptococcal) and two gram-negative coccal species. In addition, we also tested three wild-type isolates which under the light microscope appeared clearly coccoid and were identified as *Acinetobacter calcoaceticus* (two isolates) and *Proteus mirabilis*, two species known to form coccobacilli and rods of various lengths, respectively. In all gram-negative strains, septum formation was inhibited by a β-lactam antibiotic such as aztreonam, known to specifically block septum formation (45), and by two different DNA inhibitors, nalidixic acid and mitomycin (13). It is well known that DNA inhibition rapidly blocks septum formation (19). Both aztreonam and nalidixic acid are not active on most gram-positive strains (3). Therefore, in these microorganisms septum formation was inhibited by mitomycin and by methicillin (30).

Among the gram-negative organisms, both the *Proteus* and the *Acinetobacter* strains changed shape from coccoid to rod when the septum was inhibited, whereas the strains of the two different *Neisseria* species maintained their coccal shape even 3 h after septum inhibition, demonstrating at this time a cell diameter only slightly (not more than 30%) enlarged (Table 3 and Fig. 3). Among the gram-positive bacteria, all staphylococcal strains and all strains of two streptococcal species (*Streptococcus lactis* and *Streptococcus disgalactiae*) maintained their normal coccal configuration after septum inhibition, whereas the other streptococcal species underwent transition to rod shape. In all cases, all strains of the same species yielded identical responses to septum inhibition, and with all strains of all species, equal results were obtained with DNA and peptidoglycan inhibitors.

Effect of septum inhibition on cell shape of some morphol-

TABLE 3. Cell shape of wild-type cocci after inhibition of septum formation by various antibiotics

Species	Strains	Antibiotic (μg/ml)[a]	Shape after treatment for[b]:		
			60 min	90 min	240 min
P. mirabilis	VR1, VR2, VR3	Nal (20)	Rods	Long rods	Filaments
		MitC (5)	Rods	Long rods	Filaments
		Aztr (5)	Rods	Long rods	Filaments
A. calcoaceticus	VR1, VR2	Nal (20)	Rods	Rods	Rods
		MitC (5)	Rods	Rods	Rods
		Aztr (5)	Rods	Rods	Rods
N. gonorrhoeae	NG1, NG2, NG3	Nal (15)	Cocci	Cocci	Cocci
		MitC (1)	Cocci	Cocci	Cocci
		Aztr (0.1)	Cocci	Cocci	Cocci
N. catarrhalis	NC1, NC2, NC3	Nal (15)	Cocci	Cocci	Cocci
		MitC (1)	Cocci	Cocci	Cocci
		Aztr (0.1)	Cocci	Cocci	Cocci
S. aureus	VA1, VA2, VA3, VA4, VA5	MitC (5)	Cocci	Cocci	Cocci
		Met (1)	Cocci	Cocci	Cocci
S. simulans	VA1, VA2, VA3, VA4	MitC (5)	Cocci	Cocci	Cocci
		Met (1.5)	Cocci	Cocci	Cocci
S. intermedius	VA1, VA2, VA3, VA4	MitC (3)	Cocci	Cocci	Cocci
		Met (1)	Cocci	Cocci	Cocci
S. lactis	VR1, VR2	MitC (0.8)	Cocci	Cocci	Cocci
		Met (15)	Cocci	Cocci	Cocci
S. disgalactiae	VR1, VR2, VR3	MitC (1)	Cocci	Cocci	Cocci
		Met (15)	Cocci	Cocci	Cocci
S. bovis	VR1, VR2, VR3	MitC (0.8)	Coccobacilli	Rods	Long rods
		Met (15)	Coccobacilli	Rods	Long rods
S. agalactiae	VR1, VR2	MitC (1)	Coccobacilli	Rods	Rods
		Met (10)	Coccobacilli	Rods	Rods
S. faecium	VR1, VR2, VR3	MitC (1)	Coccobacilli	Rods	Rods
		Met (15)	Coccobacilli	Rods	Long rods
S. sanguis	VR1, VR2	MitC (1)	Rods	Rods	Rods
		Met (5)	Rods	Rods	Long rods

[a] The dose used corresponds to the minimal concentration inhibiting cell number increase. Nal, Nalidixic acid; MitC, mitomycin C; Aztr, aztreonam; Met, methicillin.

[b] All cells were cocci at 0 min.

ogy mutants of gram-negative rods. The relevant genetic, biochemical, and physiological properties of the mutants used are summarized in Table 4. In all strains, septum formation was blocked by aztreonam, by nalidixic acid, and by mitomycin. Of the five mutants studied, the two carrying a mutation in the *pbpA* gene that made them unable to synthesize PBP 2 maintained their coccoid configuration unalterated when the septa were inhibited, the only evident effect of inhibition being a slight increase in average cell diameter, which after 4 h incubation did not exceed 30% (Table 4). In contrast, the other two *Escherichia coli* strains, SP52 and envB, and the *K. pneumoniae* mutant, all of which continued to form an apparently normal PBP 2, even when of coccal shape, when the septum was blocked by any of the antibiotics began to elongate, undergoing complete transition to rod shape within approximately 1 h.

Effects of expressing a defect in septum formation on cell shape of coccoid morphology mutants. Similar effects can be tested only by use of double mutants in which the morphology and cell division defects are expressed under different conditions. In fact, as opposed to inability to form septa, which is expressed soon after exposure to nonpermissive conditions, expression of shape alteration (in the mutants so far described) requires prolonged exposure to the nonpermissive condition (22, 29, 36) during which cell division continues. To observe accurately the effect of mutational inhibition of the septum on shape alteration, one should first obtain the cocci and then permit expression of the septum block. Unfortunately, no such strains with well-characterized mutations exist in *E. coli*. We have therefore taken advantage of *Klebsiella* strain MIR M7, which has a pH-dependent morphology defect (formation of cocci at pH 7 and above and of rods at pH 6.4 and below) and a temperature-sensitive defect (failure to divide at 41°C). MIR M7 cells, when grown at pH 7 and at 37°C until they became cocci and then shifted to 41°C, underwent coccus-to-rod transition within 90 min (Table 5).

These findings contrast with those of others (4) who have shown that double mutants temperature sensitive for cell shape (*rodA* or *pbpA*) and for cell division (*ftsZ*) form large lemon-shaped cells at high temperature. The behavior of such double mutants may simply depend on the fact mentioned above, that in similar strains the cell division damage is fully expressed much earlier than the cell shape alteration. To test this possibility, we evaluated cell shape in the *pbpA* (SP45) and in the *rodA* (SP52) mutant when cell division was blocked by nalidixic acid either immediately after the shift to the nonpermissive temperature or after the cells had become cocci. We also analyzed the cell shape of the double mutant KJB20 (carrying the *pbpA* and *ftsZ* mutations) when this strain was grown in the presence of mecillinam (to completely block the site for lateral wall elongation) until it changed to coccal shape and then shifted to the nonpermissive temperature. Mutants SP52 and SP45 formed lemon-shaped cells when nalidixic acid was added immediately before the shift to the nonpermissive temperature (Table 5). In contrast, when the antibiotic was added after the complete transition to coccal shape, rods and unmodified cocci, as expected, were seen in SP52 and SP45, respectively. The double mutant KJB20, when pregrown in the presence of

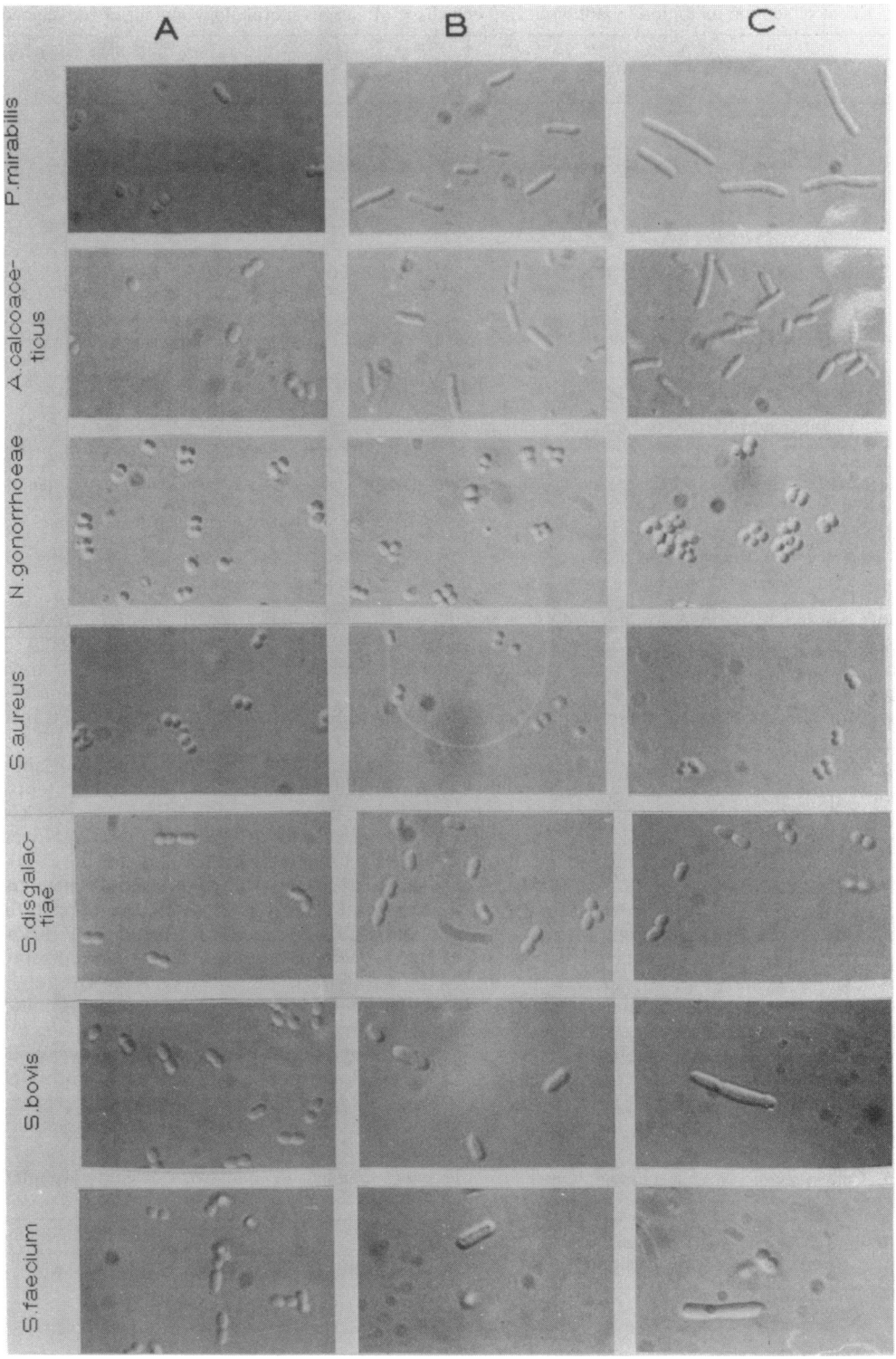

FIG. 3. Morphology of wild-type coccal strains (A) treated with a DNA synthesis inhibitor (nalidixic acid for gram-negative strains and mitomycin for gram-positive strains) (B) or a β-lactam antibiotic (aztreonam for gram-negative strains and methicillin for gram-positive strains) (C) for 120 min. Strains, antibiotics, and concentrations were as in Table 3.

mecillinam until it formed cocci at 30°C, did not form lemon-shaped cells after the shift to the nonpermissive temperature but maintained a coccal shape similar to that demonstrated before the shift. If mecillinam was removed after the transfer to high temperature, the cells, as expected, maintained their coccal shape (Table 5).

Effects of incubation under nonpermissive conditions on shape of cell growth conditional mutants isolated from wild-

TABLE 4. Effect of septum inhibition by antibiotics on cell shape of various morphology mutant of rod-shaped bacteria[a]

Strain	Relevant properties	Antibiotic (μg/ml) and growth condition[b]	Shape after drug treatment for[c]:		
			60 min	120 min	240 min
E. coli					
SP6	*pbpA* PBP2⁻, cocci at 32 and 42°C	Nal (20) at 32 or 42°C	Cocci	Cocci	Cocci
		MitC (5) at 32 or 42°C	Cocci	Cocci	Cocci
		Aztr (5) at 32 or 42°C	Cocci	Cocci	Cocci
SP45	*pbpA* PBP2⁻(Ts), cocci at 42°C	Nal (20) at 42°C	Cocci	Cocci	Cocci
		MitC (5) at 42°C	Cocci	Cocci	Cocci
		Aztr (5) at 42°C	Cocci	Cocci	Cocci
SP52	*rodA* RODA(Ts), cocci at 42°C	Nal (20) at 42°C	Rods	Rods	Rods
		MitC (5) at 42°C	Rods	Rods	Rods
		Aztr (5) at 42°C	Rods	Rods	Rods
envB	*envB* ENVB, cocci at 32 and 42°C	Nal (20) at 32 or 42°C	Rods	Rods	Rods
		MitC (5) at 32 or 42°C	Rods	Rods	Rods
		Aztr (5) at 32 or 42°C	Rods	Rods	Rods
K. pneumoniae MIR M7	Cocci at pH 7, rods at pH 5.8; temperatures sensitive for cell division	Nal (30) at pH 7	Rods	Rods	Rods
		MitC (5) at pH 7	Rods	Rods	Rods
		Aztr (5) at pH 7	Rods	Rods	Rods

[a] The strains were incubated for 120 min at 42°C before addition of antibiotics to allow full expression of altered morphology. Behavior of SP6 and envB, being constitutive mutants, was the same at both temperatures.
[b] The dose used corresponds to the minimal concentration inhibiting cell number increase. Nal, Nalidixic acid; MitC, mitomycin C; Aztr, aztreonam.
[c] All cells were cocci at 0 min.

type cocci. Mutants unable to form colonies at 42°C were isolated from one strain of as many as 10 different coccal species from among those used in the experiments described in Table 3. In all cases, the mutants were isolated from one of the strains used for the experiments mentioned above. The number of mutants isolated from each strain, with the exception of *Staphylococcus aureus* VA2, exceeded 60 and in most cases was around 100. A total of 914 mutants were isolated and analyzed. All strains were incubated at a temperature nonpermissive for colony formation, and their shape was studied at successive time intervals. None of the strains isolated from *N. catarrhalis* NC1, *S. lactis* VR2, *S. disgalactiae* VR3, *S. aureus* VA2, *Staphylococcus simulans* VA4, and *Staphylococcus intermedius* VA1, all of which maintained their coccal shape in the presence of the various septum inhibitors (Table 3 and Fig. 3), underwent any substantial shape alteration after 4 h of incubation at the nonpermissive temperature except for some staphylococcal mutants that formed clusters of coccoid cells (Table 6 and Fig. 4). In contrast, most (75 to 90%) of the thermosensitive mutants isolated from *S. agalactiae* VR1, *Streptococcus bovis* VR3, *Streptococcus faecium* ATCC 9790, and *Streptococcus sanguis* VR1, all of which formed rods in the presence of septum inhibitors, soon after the shift to the nonpermissive temperature started a transition to rod configuration which appeared complete within approximately 60 min. On continuing incubation, all mutants tended to show further elongation but to different extents in the different species. In particular, in *S. faecium* ATCC 9790, *S. sanguis* VR1, and *S. bovis* VR3, fairly long rods were observed after 120 min, whereas in *S. agalactiae* VR1 the rods were relatively short. It is interesting that in the mutants of all species that were capable of elongating at the nonpermissive temperature, parallels in elongating ability were observed in the respective parent strains when the septum was inhibited by antibiotics.

Effect on cell shape of incomplete inhibition of the septum. In strains *P. mirabilis* VR1, *A. calcoaceticus* VR1, *K. pneumoniae* MIR M7, and *E. coli* envB, doses of nalixidic acid below the MIC slowed down both DNA synthesis and

TABLE 5. Cell shape of some morphology mutants from *K. pneumonia* and *E. coli* when a defect in septum formation is expressed after or together with a defect in cell shape

Mutant strain	Antibiotic (μg/ml) and growth conditions[a]	Shape after drug treatment for:			
		0 min	60 min	120 min	240 min
K. pneumoniae MIR M7	None; pH 5.8, 37°C	Rods	Rods	Rods	Rods
	None; pH 7, 37°C	Cocci	Cocci	Cocci	Cocci
	None; pH 7, 41°C	Cocci	Rods	Rods	Rods
E. coli SP52	None; 32°C	Rods	Rods	Rods	Rods
	Nal (20) at 42°C	Rods	Large lemon	Large lemon	Large lemon
	42°C for 90 min, then Nal (20) at 42°C	Cocci	Rods	Rods	Rods
	None; 32°C	Rods	Rods	Rods	Rods
SP45	Nal (20) at 42°C	Rods	Large lemon	Large lemon	Large lemon
	42°C for 90 min, then Nal (20) at 42°C	Cocci	Cocci	Cocci	Cocci
	None; 32°C	Rods	Rods	Rods	Rods
KJB20	Mec (10) at 32°C for 90 min, then shift to 42°C	Cocci	Cocci	Cocci	Cocci
	As above, but Mec removed after 30 min at 42°C	Cocci	Cocci	Large cocci	Large cocci

[a] Morphology of cells incubated under each condition is presented in Fig. 5. Nal, Nalidixic acid; Mec, mecillinam.

TABLE 6. Effect of nonpermissive temperature on cell shape of mutants isolated from gram-positive and gram-negative cocci of various species as unable to form colonies at 42°C

Strain	No. of mutants isolated	Shape after incubation at the nonpermissive temperature for[a]:		
		60 min	120 min	240 min
N. catarrhalis NC1	65	Cocci (100)	Cocci (100)	Cocci (100)
S. lactis VR2	100	Cocci (100)	Cocci (100)	Cocci (100)
S. disgalactiae VR3	110	Cocci (100)	Cocci (100)	Cocci (100)
S. agalactiae VR1	93	Rods (80)	Rods (80)	Rods (80)
		Cocci (20)	Cocci (20)	Cocci (20)
S. bovis VR3	90	Rods (83)	Long rods (83)	Long rods (83)
		Cocci (17)	Cocci (17)	Cocci (17)
S. faecium ATCC 9790	115	Rods (75)	Long rods (75)	Long rods (75)
		Cocci (25)	Cocci (25)	Cocci (25)
S. sanguis VR1	84	Rods (90)	Long rods (90)	Long rods (90)
		Cocci (10)	Cocci (10)	Cocci (10)
S. aureus VA2	25	Cocci in clusters (68)	Cocci in clusters (68)	Cocci in clusters (68)
		Cocci (32)	Cocci (32)	Cocci (32)
S. simulans VA4	171	Cocci (52)	Cocci (52)	Cocci (52)
		Cocci in clusters (48)	Cocci in clusters (48)	Cocci in clusters (48)
S. intermedius VA1	61	Cocci (56)	Cocci (56)	Cocci (56)
		Cocci in clusters (44)	Cocci in clusters (44)	Cocci in clusters (44)

[a] Number in parentheses is percentage of mutants showing the morphology indicated. All cells were cocci at 0 min.

the rate of cell number increase by no more than 30% within the first 150 min and only slightly more during the following 30 min (Fig. 5). During this period of time, the four strains underwent approximately three cell division cycles. In the presence of these subinhibitory concentrations of nalidixic acid, the cocci of all four microorganisms underwent complete transition to rod shape within 60 min (Fig. 6). After this time, they did not elongate further but continued to proliferate as rods for an additional 120 min.

In another set of experiments, some of the thermosensitive mutants described in Table 6 were first incubated briefly at 42°C and then shifted back to the permissive temperature, after which their shape and cell division were analyzed. The shape of all mutants that remained coccoid after prolonged incubation at 42°C was not altered either during the brief exposure at the nonpermissive temperature or during regrowth at 32°C (Table 7). In addition, these mutants started to dividing again soon after the return to the permissive temperature. In contrast, in all mutants that acquired a rod shape at 42°C, a 20-min incubation at the nonpermissive temperature caused a long delay in division after the shift to 32°C. During this delay, the cells elongated and assumed a clear rod shape within approximately 60 min. After this time, several septa began to be formed in the rod-shaped cells; within approximately an additional 40 to 50 min the septa were completed, leading to short chains of cocci which soon separated, causing an increase in cell number.

Effects of a specific block of lateral wall elongation on ability of cocci to assume a rod shape after septum inhibition. Because of the lack of both antibiotics and well-characterized mutations blocking lateral wall elongation in gram-positive bacteria, at present such effects can be evaluated only in the gram-negative cocci that assume a rod shape after septum inhibition. When the wild-type cocci of strains *A. calcoaceticus* VR1 and *P. mirabilis* VR1 or cocci of mutants *E. coli* SP45 and *K. pneumoniae* MIR M7 were treated with mecillinam, a specific inhibitor of lateral wall elongation (25, 33), before septum inhibition by nalidixic acid, none of the strains either elongated or changed shape (Table 8). Indeed, they all maintained their coccal shape even after 2 h, when they appeared as regular cocci with a 30% wider diameter. Under similar conditions, *E. coli* mutant SP52 also did not elongate or change shape, but the cocci tended to enlarge with time.

Analysis of PBPs of coccus-shaped species that do and do not undergo transition to rod shape. We analyzed PBPs of different species of streptococci, some with two sites for peptidoglycan synthesis and others with only one site. Those unable to elongate (*S. lactis* and *S. disgalactiae*) carried a maximum of four PBPs, whereas those which elongated after septum inhibition (*S. agalactiae*, *S. bovis*, *S. faecium*, and *S. sanguis*) carried a minimum of six PBPs (Fig. 7). PBPs of the other species, some strains of which were used in this study, were analyzed previously by others. All species used in this study whose strains proved capable of forming the lateral wall, such as *P. mirabilis* (17), *A. calcoaceticus* (17), *K. pneumoniae* (33), and *E. coli* (17, 41), always carried at least six PBPs, whereas all species such as *Neisseria gonorrhoeae* (14), *N. catarrhalis* (see also Fig. 7), *S. aureus* (12), *S. simulans* (12), and *S. intermedius* (12) whose strains were unable to form a lateral wall always carried fewer PBPs (never more than four) (Table 9).

DISCUSSION

The goal of this study was to evaluate a number of predictions made possible by the TCS model for shape regulation in bacteria. These predictions specifically concern the effects of septum inhibition, either by antibiotics or by mutations, on cell shape of wild-type cocci and of coccal cells of morphology mutants. The predictions tested have all been fulfilled.

The model predicts (see also Fig. 2) that there are two major types of wild-type cocci and morphology mutants with coccal shape. In the first type, a round shape depends on the fact that bacteria carry (in a functional form) only the site for septum formation, either because another site has never been generated (wild-type cocci) or because a mutation has destroyed the site for lateral wall elongation (mutants with coccal shape). In contrast, cocci of the other type carry the two competing sites, but their activity is not balanced (in

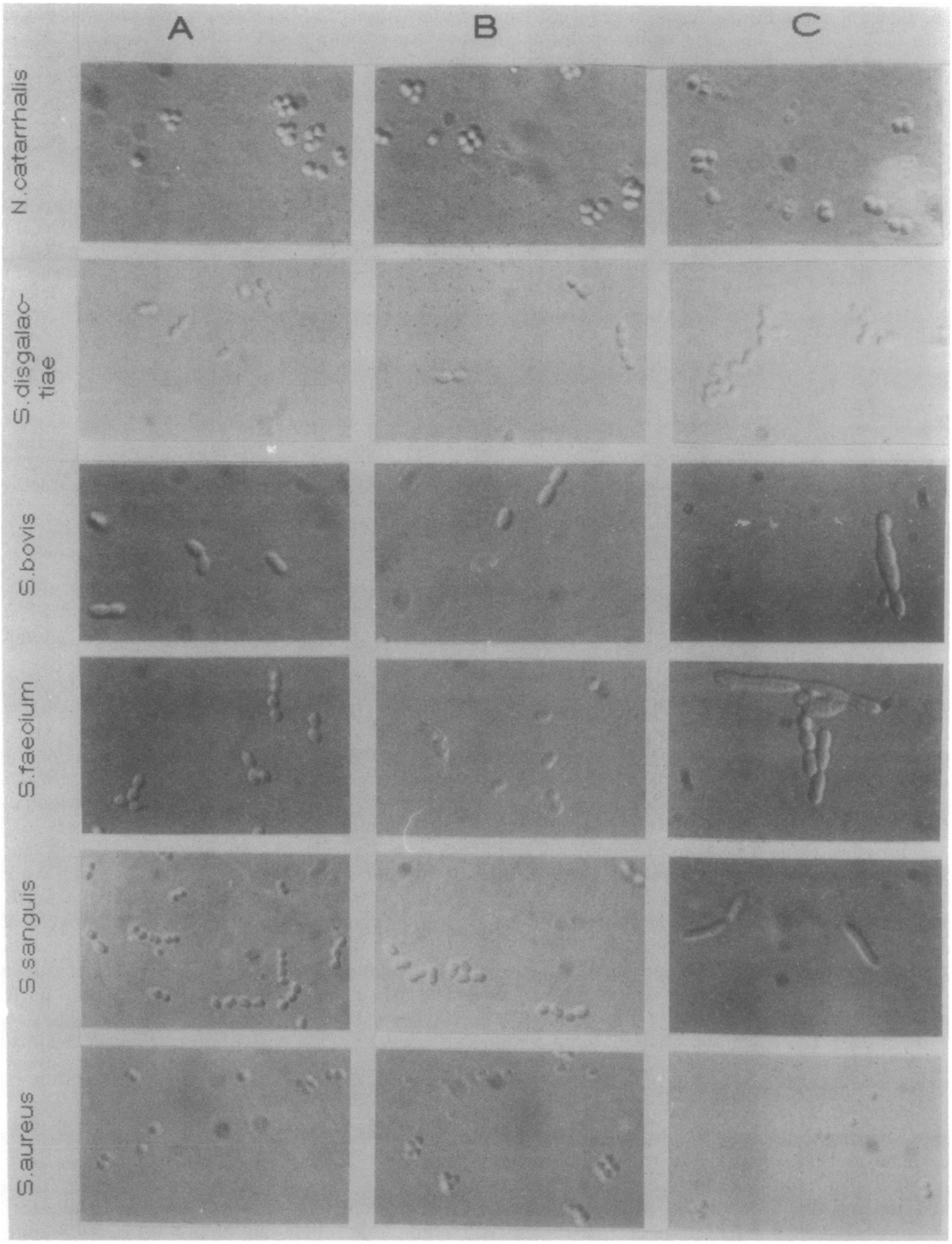

FIG. 4. Morphology of thermosensitive cell growth mutants from different coccal species incubated at 32°C (A) or at 42°C (B and C) for 60 min. One mutant that maintained its coccal shape at 42°C and one mutant that changed shape at 42°C, isolated from *S. bovis*, *S. faecium*, and *S. sanguis*, are shown in panels B and C, respectively. Two mutants isolated from *N. catarrhalis*, *S. disgalactiae*, and *S. aureus* and are also shown in panels B and C.

wild-type cocci) or the balance of the competition remains constantly in favor of the septum because of its hyperactivity or because of an abnormally weak activity of the lateral wall formation site (in the mutants) (34). Blocking the septum should allow expression of lateral wall formation in the latter strains, which under these conditions ought to undergo transition to rod shape, but not in the first type, which should maintain their coccal shape. In this study, the effect of blocking the septum by antibiotics was evaluated in a total of 37 wild-type strains belonging to 13 species and in all of the morphology mutants so far described to which we have had access. In all cases, at least one inhibitor of DNA synthesis and one β-lactam known to specifically block the septum (when possible) were used. The effect of mutations that block septum formation was studied by analyzing as many as 914 strains of 10 species. As predicted by the model, some

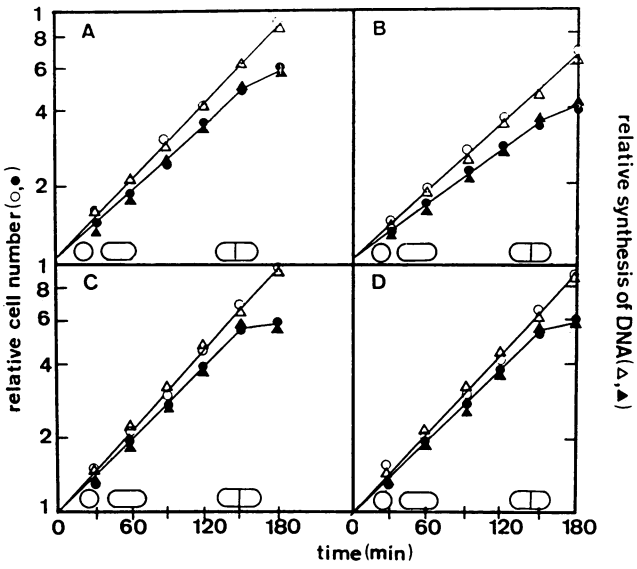

FIG. 5. Cell number increase (○, ●) and DNA synthesis (△, ▲) of *P. mirabilis* VR1 (A), *A. calcoaceticus* (B), *K. pneumoniae* MIR M7 (C), and *E. coli* envB (D) grown in the presence of subinhibitory concentrations of nalidixic acid. △ and ○, Untreated cells; ▲ and ●, cells treated with 2 (A) or 4 (B to D) μg of nalidixic acid per ml.

wild-type cocci and some coccal morphology mutants underwent transition to rod shape when the septum was blocked either by antibiotics or by mutations, whereas others maintained their coccal shape. In this connection, it is important to stress that with both wild-type strains and morphology mutants, in order to obtain the coccus-to-rod transition, antibiotics and mutations did not have to cause complete block of the septum; temporary inhibition (with mutations) or incomplete inhibition (with antibiotics) was sufficient to make both wild-type and mutant cocci grow as rods. This finding is in full accord with the peculiar dynamic feature of the model, whereby cell elongation and cell division are determined by the equilibrium between two competing reactions, and therefore any interference (whether stimulatory or

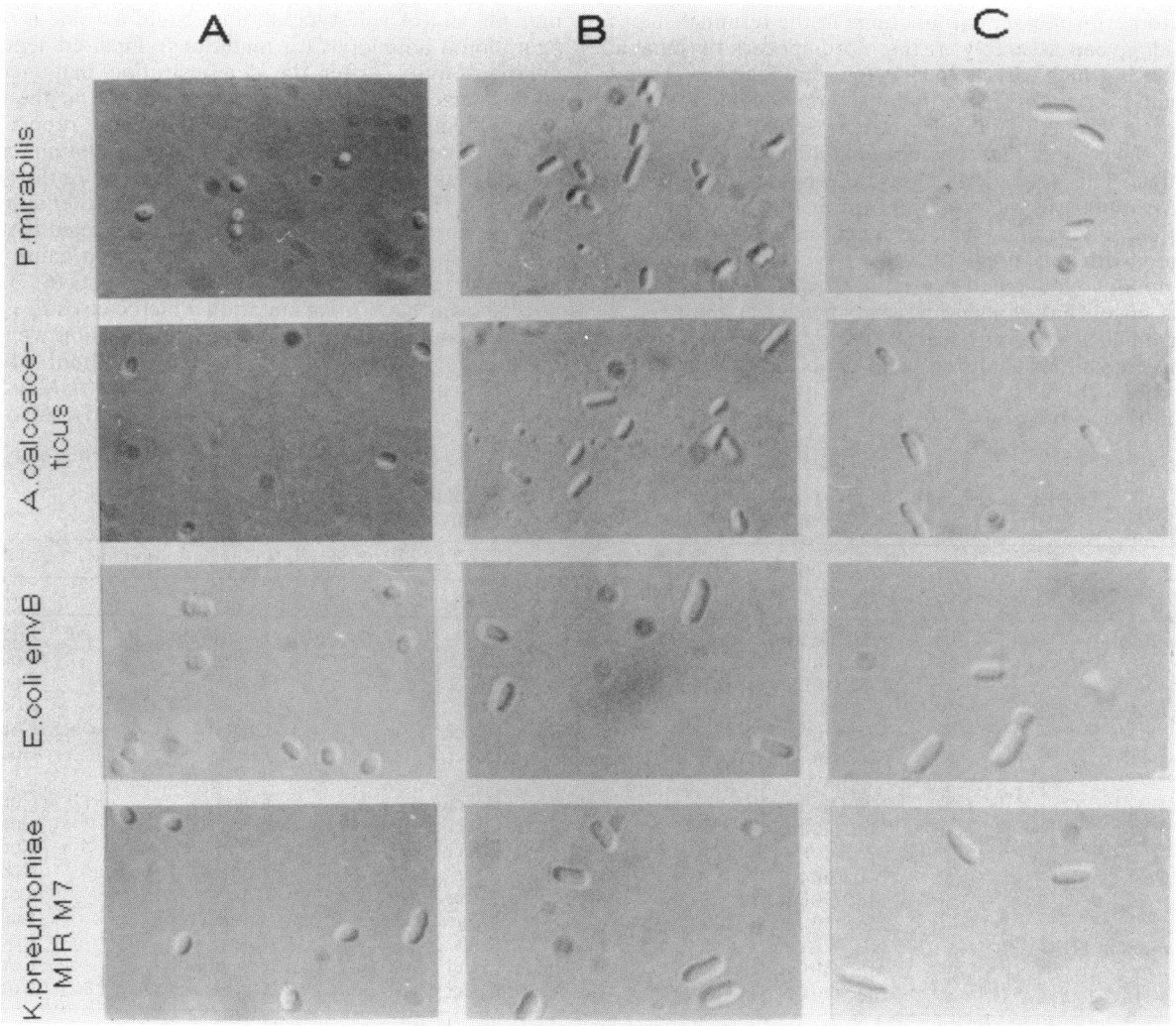

FIG. 6. Morphology of *P. mirabilis* VR1, *A. calcoaceticus* VR1, *K. pneumoniae* MIR M7, and *E. coli* envB untreated (A) and grown in the presence of subinhibitory concentrations of nalidixic acid for 90 (B) and 180 min (C) Antibiotic concentrations were the same as for Fig. 5.

TABLE 7. Effect of preincubation at 42°C on cell shape and division at the permissive temperature of some mutants thermosensitive for colony formation

Mutant strain	Shape					Cell no. increase (%) at 32°C after[a]:		
	In the presence of β-lactam[b]	After 60 min at 42°C	At the time of shift[c]	At 32°C after[d]:				
				60 min	120 min	60 min	120 min	180 min
S. lactis ML3	Cocci	Cocci	Cocci	Cocci	Cocci	55	140	290
S. disgalactiae MD5	Cocci	Cocci	Cocci	Cocci	Cocci	60	126	300
S. agalactiae MA37	Rods	Rods	Coccobacilli	Rods	Septated rods	1	19	290
S. bovis MB71	Rods	Rods	Coccobacilli	Rods	Septated rods	3	20	260
S. faecium NON-11	Rods	Rods	Coccobacilli	Rods	Septated rods	2	30	295
S. sanguis MS80	Rods	Rods	Coccobacilli	Rods	Septated rods	0	25	255
S. aureus ts-6	Cocci	Cocci	Cocci	Cocci	Cocci	48	150	340
S. simulans ts-12	Cocci	Cocci	Cocci	Cocci	Cocci	50	170	345

[a] Calculated with respect to the time of the temperature shift.
[b] The doses of antibiotic used are indicated in Table 3.
[c] The shift from 42 to 32°C was made after 20 min of incubation at the nonpermissive temperature.
[d] All cells were cocci after 180 min.

inhibitory) with either of the two reactions which changes their equilibrium also alters expression of the two competing reactions.

Support for the TCS model has also been provided by analysis of PBPs of the strains used in this study. PBPs are known to perform essential functions in the terminal stages of peptidoglycan assembly. It therefore appears reasonable that bacteria which carry two sites for peptidoglycan assembly should carry more PBPs than bacteria which carry only one site. Moreover, this characteristic should be independent of the shape that bacteria exhibit under ordinary conditions. The cocci that elongate when the septum is blocked should carry more PBPs (probably similar to the number carried by rods) than do cocci that do not elongate. In accord with this prediction, we have found that cocci which do not elongate carry a maximum of four PBPs, whereas cocci that elongate never carry fewer than six, a number equal to that of bacteria that grow as rods. Apart from the predictions analyzed here, the basic concept peculiar to the TCS model allowed us to make other unique predictions that were also not possible outside the framework of the model. We were able to predict, before any suggestion or experimental finding by others indicating similar possibilities, that the inability to form septa could be overcome by antibiotics (or mutations) interfering with lateral wall elongation and that cocci, both of morphology mutants and of wild-type strains, could assume a rod configuration if antibiotics (or mutations) impaired septum formation activity. After the demonstration that mecillinam allows some cell division mutants to divide at the nonpermissive temperature and after a preliminary report of ours showing that antibiotics that interfere with septum formation allow certain morphology mutants to reacquire their normal rod shape (10, 34), others have provided further support for these predictions. Some authors have confirmed that antibiotics which interfere with septum formation cause elongation of some but not other morphology mutants (6, 7). Others have shown that a *rodA* mutation which causes *E. coli* cells to assume a coccal shape restores cell division when introduced into bacteria carrying an *ftsI* mutation, whereas a mutation causing inhibition of cell division (*ftsI23*) corrects the shape damage of *rodA* (*sui*) mutants (5). Also clearly

TABLE 8. Effect of septum formation and lateral wall extension inhibition on morphology of coccal strains and mutants isolated from gram-negative species

Strain	Antibiotic (μg/ml) and growth condition[a]	Shape after drug treatment for[b]:		
		60 min	120 min	240 min
P. mirabilis VR1	Nal (20) at 37°C	Rods	Long rods	Filaments
	Mec (10) at 37°C	Cocci	Cocci	Cocci
	Mec (10) + Nal (20) at 37°C	Cocci	Cocci	Cocci
A. calcoaceticus VR1	Nal (20) at 37°C	Rods	Rods	Rods
	Mec (10) at 37°C	Cocci	Cocci	Cocci
	Mec (10) + Nal (20) at 37°C	Cocci	Cocci	Cocci
E. coli				
SP45	Nal (20) at 42°C	Cocci	Cocci	Cocci
	Mec (10) at 42°C	Cocci	Cocci	Cocci
	Mec (10) + Nal (20) at 42°C	Cocci	Cocci	Cocci
SP52	Nal (20) at 42°C	Rods	Rods	Rods
	Mec (10) at 42°C	Cocci	Cocci	Cocci
	Mec (10) + Nal (20) at 42°C	Large cocci	Large cocci	Large cocci
K. pneumoniae MIR M7	Nal (30) at pH 7	Rods	Rods	Rods
	Mec (10) at pH 7	Cocci	Cocci	Cocci
	Mec (10) + Nal (30) at pH 7	Cocci	Cocci	Cocci

[a] When two antibiotics were used, nalidixic acid (Nal) was added to the cultures only after mecillinam (Mec) and the nonpermissive temperature had induced the complete transition to coccal shape.
[b] All cells were cocci at 0 min.

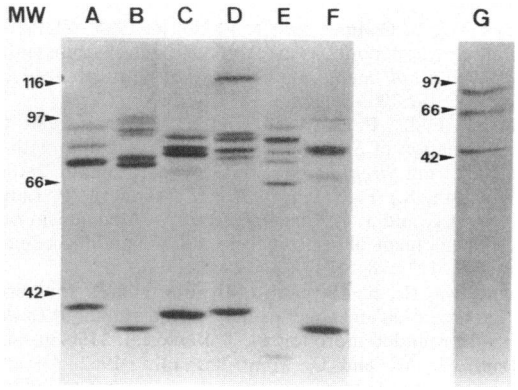

FIG. 7. Electrophoretic patterns of PBPs of *S. lactis* (lane A), *S. bovis* (lane B), *S. sanguis* (lane C), *S. faecium* (lane D), *S. agalactiae* (lane E), *S. disgalactiae* (lane F), and *N. catarrhalis* (lane G).

predicted was the possibility, recently demonstrated as correct, that hyperactivity of the site for lateral wall elongation, possibly as a result of increased dosage of a gene (*mreB*) involved in the process of lateral wall formation, caused inhibition of cell division and formation of filaments (49).

We found these findings neither surprising nor innovative conceptually in that some of the predictions they confirm had been stated a long time ago (33). These findings are clearly explained by the TCS model. Probably reference to this hypothesis would had been of help in previous interpretations. It comforted us to read of these experiments, which in several cases appeared to confirm our own previous work, repeating, in different experimental systems, experiments that we ourselves performed some time ago and which deserved citation and perhaps some measure of credit. What we did find surprising, however, was the fact that the authors ascribed to a hypothesis other than the TCS model (5) the concept whereby the activity of the sites for lateral wall elongation and for septum formation alternates during the cell cycle. No trace of this concept can be found in the paper cited (20), and it cannot be taken for granted that such activity alternates, as documented by the fact that some authors disagree with the concept and, in doing so, cite the TCS model (9, 51).

TABLE 9. Morphology and PBPs of species used

Species	Morphology when septum formation is inhibited	No. of PBPs in membrane	Reference(s)
P. mirabilis	Filament	6/7	17
A. calcoaceticus	Rod	6/7	17
E. coli	Rod or filament	6/7	17, 41
K. pneumoniae	Rod	6	33
N. gonorrhoeae	Coccus	3	14
N. catarrhalis	Coccus	3	This study
S. aureus	Coccus	4	12
S. simulans	Coccus	3	12
S. intermedius	Coccus	3	12
S. lactis	Coccus	4	This study
S. disgalactiae	Coccus	4	This study
S. bovis	Rod	7	This study
S. agalactiae	Rod	6	This study
S. faecium	Rod	6	16
S. sanguis	Rod	6	This study

The power of the TCS model to predict experimental observations that are highly unexpected in light of conventional views regarding mechanisms of cell shape regulation and division has allowed us to make other additional original observations. We have demonstrated in this report that two types of wild-type cocci exist; one possesses two or three additional PBPs and has the potential to form rods, whereas the other has no such potential.

This is the first report describing mutants isolated from wild-type cocci that undergo transition from coccal to rod shape. Many mutants with altered shape have been described previously in both gram-negative and in gram-positive bacteria, but in all cases the mutants were isolated from rod-shaped species and mutations led to the formation of cocci instead of normal rods (1, 8, 22, 25–27, 31, 40, 42, 50). In contrast, the possibility of obtaining the rod shape as a consequence of a mutation occurring in wild-type cocci has never even been considered before, probably because since the rod shape involves a higher organizational complexity than does the coccal shape, it was thought that additional genes not carried by cocci were needed to obtain rod-shaped bacteria (20). In contrast, as described above, the TCS model also considers the possibility that wild-type bacteria may ordinarily grow as cocci even when they carry the site for lateral wall elongation, provided that the activity of the site is relatively low (34). In these species, a single mutation can shift the balance of the competing reactions in favor of lateral wall extension (either by impairing the septum or by increasing the lateral wall activity) and cause the formation of rods.

This is also the first time that cell division inhibitors have been shown to cause formation of rods in the wild-type cocci of some, but not other, bacterial species, which previously had never been regarded as possible. In addition, we demonstrate here for the first time that in a cell in which the expression of a first mutation has caused loss of normal shape, the correct rod shape can be regained by expression of another mutation that interferes with ability to form septa. This, then, may be a promising experimental system for showing the role of interactions between the sites for lateral wall elongation and septum formation in determining bacterial cell shape. These findings, particularly when taken together with those obtained with mutants of wild-type cocci, with those observed with antibiotics that did not completely block cell division, and with those obtained upon treating temperature-sensitive cell division mutants with mecillinam, strongly support the basic concept of the TCS model and provide the logical and likely explanation for the findings of other authors (5, 49) with respect to the two competitive sites.

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