

Gelatin-induced Reversion of Protoplasts of *Bacillus subtilis* to the Bacillary Form: Biosynthesis of Macromolecules and Wall During Successive Steps¹

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Protoplasts of *Bacillus subtilis* plated on SDG medium formed L colonies in quantitative yield and propagated in the L-form indefinitely. Protoplasts or L bodies placed in 25% gelatin medium formed bacillary colonies. Details of the reversion of these naked bodies to the walled form are reported here. Protoplasts prepared in minimal medium reverted fairly synchronously 3 to 4 hr after inoculation into gelatin, but protoplasts preincubated in casein hydrolysate (CH)-enriched minimal medium were primed to revert within 1 hr in the gelatin. Preincubation for 1.5 hr in 0.44% CH was required for good priming. Cells must be subjected to this preincubation (step 1) in the naked state; it is effective for L bodies as well as protoplasts. Priming was blocked by chloramphenicol, puromycin, and actinomycin D but was not affected by penicillin, lysozyme, or inhibition of deoxyribonucleic acid (DNA) synthesis. It is concluded that protein and ribonucleic acid (RNA) synthesis are required during step 1, that DNA synthesis is not required, and that wall mucopeptide is not made. The reversion of well-primed protoplasts in the gelatin (step 2) proceeded undisturbed in thymine-starved cells with chromosomes arrested at the terminus. It was scarcely slowed by chloramphenicol in the gelatin but was delayed about 3 hr by both puromycin and actinomycin D. Escape from inhibition occurred while the inhibitors were still actively blocking growth. Penicillin and cycloserine inhibited and lysozyme reversed reversion. Momentary melting of the gelatin delayed reversion. It is concluded that mucopeptide synthesis occurs in step 2, that concomitant RNA, DNA, or protein synthesis is not essential, but that physical immobilization of excreted cell products at the protoplast surface is necessary early in step 2. Newly reverted cells were misshapen and osmotically sensitive. Processes which confer osmotic stability after reversion (step 3) did not occur in the presence of chloramphenicol or actinomycin D.

When the wall of a bacterial cell is partly damaged by incubation with penicillin in hypertonic medium, or by treatment with lysozyme, it is ordinarily repaired promptly once the antibiotic or enzyme is withdrawn (1, 17). However, if all of the wall is stripped away and (naked) protoplasts are formed, synthesis of new wall does not restart when penicillin or lysozyme is removed. When kept in liquid medium, the protoplasts merely enlarge but do not divide. In protein-containing soft agar, however, such naked cells often

give rise to L colonies, thus manifesting their inability to initiate wall formation through successive cell generations. Depending on the bacterial species, these L colonies may never show reversion to the walled bacillary state [e.g., in the penicillin-induced L-forms of "small-colony" mutants of *Escherichia coli* (11)], or, at the opposite extreme, reversion may occur when the L colonies have barely begun to grow in the soft agar [e.g., in lysozyme protoplasts of *Bacillus megaterium* (10, 11)]. *B. subtilis* shows an intrinsic reversion proclivity intermediate between that of the small-colony mutants of *E. coli* and that of *B. megaterium*. As a result of this moderate intrinsic tendency to revert, this bacterium has

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proven very useful in studies of reversion. Preceding papers on this organism have documented the reversion-inhibiting effect of several D-amino acids (12) and of high NaCl, NaNO₃, and KCl concentrations, a requirement for Mg⁺⁺ and K⁺ ions for reversion (13), and the crucial importance of the physical characteristics of the medium in determining the occurrence of reversion. In particular, reversion was found to be virtually absent in liquid media, slow in soft agar, and markedly stimulated by gelatin and hard-agar media (12), by membrane filters (11), by cell wall fractions, and by a great variety of autoclaved intact microorganisms (D. Clive, Ph.D. Thesis, Georgetown Univ., Washington, D.C., 1968). Among these various environments, 25% gelatin media induce reversion most efficiently, and, hence, gelatin-induced reversion has been studied in greatest detail. Photomicrographic and electron-micrographic and physical studies of gelatin-induced reversion have been published (13, 16). In this paper, successive phases in gelatin-induced reversion are described, along with details of wall and macromolecular biosyntheses during these phases.

Reversion has been studied quantitatively in only a few microorganisms besides *B. subtilis*. In *Streptococcus faecalis* strains, reversion is also stimulated by gelatin and hard agar and is inhibited by 0.43 M NH₄Cl (5). Yeast protoplasts likewise are efficiently induced by gelatin to revert to the walled form (18, 19). This parallelism between yeast and bacterial protoplasts is especially noteworthy because the chemical constitution of the yeast cell wall is quite different from that of bacteria.

MATERIALS AND METHODS

Strains. *B. subtilis* strain 168 (tryptophan-requiring) was used for most experiments; strain SB566 (tryptophan- and thymine-requiring) was employed in the experiments concerned with the role of deoxyribonucleic acid (DNA) synthesis in reversion.

Media. The media used were slightly modified versions of earlier ones (12, 13, 17).

SDG plating medium contained the following ingredients, per liter: 8 g of agar (Difco); 1 g of NH₄NO₃; 0.1 g of (NH₄)₂SO₄; 3.5 g of K₂HPO₄; 1.5 g of KH₂PO₄; 2 g of glucose; 0.02 g of tryptophan; 0.6 g of D-methionine; 20 g of gelatin (Difco); 0.5 M sodium succinate; 0.005 M MgCl₂.

SDS medium was used interchangeably with SDG medium; it differed from it only in that 20 g of gelatin per liter was replaced with 5 ml of human serum or horse serum per liter. These two media were used throughout to analyze cell mixtures for the presence of L colony-forming cells and bacillary colony-forming cells. Because of the presence of the reversion-inhibitor D-methionine, L colonies were stable on these

media and were not overgrown by bacillary revertants.

In virtually all experiments, DP medium (13) or A medium was used in parallel with the rather similar SDG or SDS medium. A medium was the same as DP medium except that it contained 1 g of acid-hydrolyzed casein (Nutritional Biochemicals Corp) per liter instead of 5 g. DP and A media permitted rapid growth of bacillary and L colonies, but results had to be evaluated within 48 hr of inoculation, because otherwise revertants began to overgrow the L colonies.

S⁻ medium was the same as SDG medium except that it contained no succinate and no D-methionine. Osmotically sensitive bacilli or protoplasts did not form colonies on S⁻ medium.

Blood Agar Base (Difco) was used to grow vegetative cell inocula; Nutrient Agar (Oxoid) was used to maintain stocks in the spore state; Brain Heart Infusion (Difco) was used as an alternative to S⁻ medium; and the gelatin reversion medium, GFR, was the same as GR medium (13), except that MgSO₄ replaced MgCl₂.

SFL2, a liquid medium used in the preparation of protoplasts, contained the following ingredients, per liter: 14 g of K₂HPO₄, 2 g of KH₂PO₄, 2 g of (NH₄)₂SO₄, 1 g of sodium citrate·2H₂O, 1.43 g of MgSO₄·7H₂O, 5 g of glucose, 5 × 10⁻⁶ M ethylenediaminetetraacetate (EDTA), and 0.5 M sucrose [modified SL2 medium (12)]. Several other media were used which were identical to SFL2 medium except that the concentrations of casein hydrolysate (CH), tryptophan, and sucrose were varied. These modifications and their designations are listed below: SFL2 contained no CH, no tryptophan, and 0.5 M sucrose; SFL3 contained 0.43% CH, 0.008% tryptophan, and 0.67 M sucrose; SFL4 contained no CH, no tryptophan, and 0.67 M sucrose; SFL5 contained 0.02% CH, 0.001% tryptophan, and 0.67 M sucrose.

Chemicals. Actinomycin D was a gift from E. Katz; puromycin was donated by M. Yarmolinsky; chloramphenicol and penicillin G were products of Parke, Davis & Co. Cycloserine was purchased from Nutritional Biochemicals Corp.

Preparation of protoplasts for reversion experiments. The initial phases of inoculation and cell growth were the same as described earlier (13). After 4 to 4.5 hr of growth in SL1 medium (12) the bacilli were diluted 1:10 into SFL2 medium to a cell density of about 10⁸/ml, incubated for an additional 1.5 hr, and then frozen for at least 10 min by immersion in a dry ice-acetone mixture.

Reversion in gelatin tubes. A sample of appropriate size was withdrawn from deep freeze (-20 C) storage, melted under the cold water tap, and incubated for 30 min at 37 C with shaking. The suspension was then diluted 1:5 into fresh medium so that the density was about 2 × 10⁷/ml and the final medium composition was that of SFL3 (or SFL4). A viable count was made (with SFL4 as dilution fluid), and the suspension was dispensed into Erlenmeyer flasks in shallow layers (e.g., 5 ml in 125-ml flasks). Lysozyme was then added to 250 μg/ml, and the flasks were incubated for 90 min without agitation in a water bath at 33 C. (This we call step 1 incubation.) To measure survival

after protoplasting, a 5×10^6 dilution was plated on SDG or similar medium, with SFL4 as dilution fluid. Since viability varied, survival figures are given in the footnotes to the tables describing the individual experiments. To measure completeness of protoplasting, a 10^8 dilution was plated on SDG medium. Only a small number of residual bacillary colony-forming cells were found after the 90-min incubation period; this number averaged 2 per 10,000 L colony-forming cells in 22 experiments and 4 per 10,000 was exceeded in only one case.

After the 90-min, step 1 incubation, the protoplast suspension was diluted 1:10 into SFL4 medium, and 0.05 ml of this suspension (50,000 to 100,000 protoplasts) was pipetted into 1 ml of GFR medium in a graduated conical centrifuge tube held at 40 C. (The GFR medium was dispensed at 55 C from a warmed wide-mouth pipette. To control the volume of the viscous medium dispensed, graduated tubes were used to receive it.) The inoculum was carefully and completely mixed into the gelatin with a wire loop, avoiding bubbles. Concentrated (20 \times) solutions of inhibitors were also added at this time when appropriate. When all tubes of a series had been stirred, they were transferred to a water bath at 26 C to begin step 2 incubation. Immediately after the gelatin had solidified, two tubes were removed for zero time sampling; this serves as a double check on survival and on the presence of bacillary forms. The gelatin was melted in a water bath at 40 C, 4 ml of prewarmed SFL4 was added, and the mixture was stirred until mixing was complete. A subsample of the warm suspension was again diluted 1:5 with SFL4, and 0.1-ml volumes were plated in duplicate or, more usually, in triplicate on SDG (or SDS) and on A (or DP) media. Ordinarily there were 100 to 350 colonies on each plate. Subsequent samples were handled in the same way as the zero time sample. Since neither the inoculum protoplasts nor the bacillary revertants divided during the reversion period (13; and see below), the total number of L colonies counted at zero time was similar to the combined total of bacillary and L colonies monitored by later platings. All plates were incubated at 30 C or at room temperature.

A variation of the preceding procedure was to prepare washed, frozen protoplasts by the method of Clive (Ph.D. Thesis, Georgetown Univ., Washington, D.C., 1968, protoplasting done in a medium containing 0.01% CH), and to start step 1 incubation with thawed protoplasts (used only in experiment of Table 2).

Preparation of L body suspension. A plate of SDG medium was inoculated with several tens of thousands of protoplasts. After 3 days of incubation at 30 C, the plates were surveyed with the dissecting microscope, and possible reverting colonies were removed by a capillary tube connected to the vacuum line. The plate was then flooded with 5 ml of SFL4, and the L growth was loosened with a spreader. The mixture of fluid, agar and L colonies was pipetted off and passed four times through a 20-gauge syringe needle to break up the L colonies. The suspension was then centrifuged at 1,500 rev/min for 10 min to remove agar particles and L-body clumps. The supernatant suspension was

frozen. A sample was melted and assayed for viable count. Later, appropriate dilutions of the melted suspension were used as inoculum in a gelatin tube experiment.

RESULTS

Step 1 (preincubation): presence of CH. Electron-microscopic, photomicrographic, and physical studies of gelatin-induced reversion (13, 16) have shown that reversion occurs fairly synchronously in protoplast populations 4 to 6 hr after incubation into gelatin reversion medium. In crowded cultures, the sudden increase in the number of revertants is delayed longer and occurs 9 or even 17 hr after incubation.

Recently it was observed that the presence of high concentrations of CH during protoplasting results in much more rapid reversion when the protoplasts are subsequently tested in the gelatin-tube system. This observation was the starting point of the present study. Early experiments were designed to specify more precisely the requirements for the CH stimulation of reversion. Table 1 shows the effect of varying the duration of the protoplasting incubation (step 1) in the high-CH medium, SFL3. Evidently, a marked boost in the reversion rate was produced when incubation in SFL3 was extended to 90 min or more; longer incubations produced a further increase.

In the experiments shown in Table 2, the effect of varying the CH concentration was examined. Washed protoplasts prepared in low (0.01%) CH medium and stored in the frozen state (D. Clive, Ph.D. Thesis, Georgetown Univ., Washington, D.C., 1968) were used. Even 0.43% CH was insufficient to give the maximal reversion rate. Nevertheless, 0.43% CH and a step 1 incubation period of 90 min were somewhat arbitrarily adopted as standard for subsequent experiments. Accepting the proposition that preincubation in high-CH medium accelerates reversion, the question arose as to whether the exposure of cells to high CH must take place in the protoplast state or whether bacilli incubated in high-CH medium and then stripped of their wall would revert equally well. To test this question, the following experiment was performed (Table 3). A culture in SFL3 medium was divided in two at the start of the step 1 procedure. To one half, lysozyme was added at zero time and incubation was continued for 120 min. This culture was thus exposed to high CH during and after protoplasting. To the other half, lysozyme was added at 90 min and incubation was continued to 120 min. This culture had the same total exposure to high CH, but only 30 min of this time in the protoplast state. Table 3 shows that the latter population was not nearly so well primed to revert as the

TABLE 1. Step 1: effect of duration of preincubation in high-CH media upon subsequent reversion in gelatin

Expt	Time in SFL3 ^a	Per cent reversion in 1 hr ^b	In gelatin after	
			2 hr	3 hr
1 ^c	<i>min</i>			
	45		10	27
		3	79	30
	90		83	85
		6	88	89
	135		89	
	180	22	92	
		21		
2 ^c	30		2	
	50		9	49
	90		41	90
	120		78	93

^a SFL3 medium contains 0.43% CH.

^b Each figure represents one gelatin tube.

^c In Experiment 1, 69% of the cells survived protoplasting and 85% survived in experiment 2.

TABLE 2. Step 1: effect of variations in CH concentration upon subsequent reversion in gelatin^a

CH in preincubation medium ^b	Per cent reversion in gelatin in				
	Expt 1		Expt 2		
	1 hr	2 hr	0.5 hr	1 hr	2 hr
%					
0	7 ^c	77 ^c	8 ^c	7 ^c	
	6		0.4	6	
0.43	17	88	0.1	19	61
	19		0.5	23	68
1.0	44	84	7	60	73
	39		10	79	80

^a An inoculum of washed protoplasts, stored in the frozen state, was used. These protoplasts were prepared in a medium containing 0.01% CH by the method of Clive (Ph.D. Thesis, Georgetown Univ., 1968).

^b Preincubation was for 90 min.

^c These reversion figures are higher than usual for reversion of unprimed protoplasts. Freezing-thawing may account for this.

former. Exposure to high CH thus must be given in the protoplast state.

Step 1 (preincubation): effect of chloramphenicol, puromycin, or actinomycin on subsequent reversion. Two alternative explanations of the preceding results are that amino acids are needed during preincubation to form wall peptide or that protein synthesis is required during step 1 to prime cells

to revert during step 2 in gelatin. Inhibitors of protein and ribonucleic acid (RNA) synthesis should be effective during preincubation only in the latter case. Table 4 shows a pertinent representative experiment. It is clear that chloram-

TABLE 3. Step 1: effect of exposure to high CH in bacillary and protoplast states upon subsequent reversion in gelatin

Sample	Incubation in SFL3		Per cent reversion in gelatin in			
	Time of lysozyme addition	Time of transfer to gelatin	Expt 1 ^a		Expt 2 ^b	
			1 hr	2 hr	1 hr	2 hr
	<i>min</i>	<i>min</i>				
1 ^c	0	30	4	0.6		
2	0	120	89	94	42	65
			85	96	26	74
3	90	120	12	48	8	11
			8	36	4	15

^a Survival after protoplasting was 34% in sample 1, 51% in sample 2, and 52% in sample 3.

^b Survival after protoplasting was 96% in sample 2 and 67% in sample 3.

^c Control subexperiment showing that 30 min of protoplasting exposure to high CH is insufficient to prime reversion.

TABLE 4. Step 1: effect of chloramphenicol, puromycin, or actinomycin D preincubation on subsequent reversion

Sample	Incubation condition during step 1 ^a	Per cent reversion in gelatin ^b after		
		1 hr	2 hr	3 hr
1	Control in SFL3	88	94	89
		84	89	
2	No CH present (SFL4)	0.8	2	12
		0.7	3	
3	Chloramphenicol (20 μg/ml) in SFL3	0	5	10
		0	5	
4	Puromycin (20 μg/ml) in SFL3	0.5	0.4	2
		2	2	
5	Actinomycin D (1 μg/ml) in SFL3	0.3	18	32
		0	23	

^a Compared to the viable count of the bacillary suspension before lysozyme addition, survival after the 90-min, step 1 incubation in the various samples was as follows: (1) 40%; (2) 83%; (3) 110%; (4) 26%; (5) 58%. Note that in this experiment and in the subsequent experiments viability loss was usually no greater in the drug-containing samples than in the controls.

^b The 200-fold dilution intervening between steps 1 and 2 reduced the inhibitor concentrations to ineffective levels in the gelatin.

phenicol, puromycin, and actinomycin all sharply reduced the reversion rate; hence, it can be concluded that the reaction of step 1 which primes reversion involves protein synthesis.

As would be expected in a system requiring protein and RNA synthesis, the presence of 10^{-3} M hydroxylamine during preincubation effectively inhibited reversion in gelatin (20).

Step 1 (preincubation): effect of penicillin. Although the protoplasts formed only L colonies after 90 min of preincubation in high-CH medium and hence were presumed to possess no wall (11, 17), it was important to determine whether the presence of penicillin during step 1 would delay reversion in gelatin. The results of the three experiments which were performed to answer this question are recorded in Table 5. In two of these, penicillin in step 1 appeared to stimulate reversion in the gelatin; in the third, it had a slight inhibitory effect. Although we have no explanation for these variations, it seems fair to conclude that penicillin-sensitive steps in mucopeptide wall synthesis are not required for the development of primed protoplasts during step 1.

Step 2 (reversion): effect of chloramphenicol, puromycin, and actinomycin D. Before the effect of preincubating protoplasts in high-CH medium was recognized, experiments had shown that, generally, reversion is sensitive to chloramphenicol. The preceding experiments opened the possibility that this sensitivity might reside exclusively in step 1 events. If cells were fully primed by protein synthesis during step 1, would they still require protein synthesis in the gelatin in order to revert? In the experiments shown in Table 6, chloramphenicol, puromycin, and actinomycin D were tested for their inhibitory effects in gelatin. A peculiar pattern of inhibition was observed: at the earliest sampling, 1 hr, an inhibition was discerned with all three antibiotics; by 2 hr, the chloramphenicol-containing samples were indistinguishable from the controls, whereas in the puromycin and actinomycin D tubes reversion was still appreciably lower. This difference persisted to the 3rd hr. The general pattern of escape from inhibition shown in Table 6 and the notably more rapid escape in the case of chloramphenicol were observed regularly. A slight inhibitory effect of chloramphenicol was registered in the 1-hr sample in five of six experiments, whereas there was no inhibition in the 2-hr sample in nine of nine experiments. By contrast, 1 μ g of actinomycin D per ml gave inhibition, usually marked inhibition, in the 2-hr sample in nine of nine experiments, and in five of seven experiments escape was not yet complete after 3 hr. The escape from inhibition in these experiments is noteworthy because it occurred in the

TABLE 5. Step 1: effect of penicillin G preincubation on subsequent reversion in gelatin

Expt ^a	Incubation conditions during step 1	Per cent reversion in gelatin after		
		1 hr	2 hr	3 hr
1a	Control in SFL3		61	88 78
1b	Penicillin (1 unit/ml) in SFL3		95	97 92
2a	Control in SFL3	61	89 89	
2b	Penicillin (1 unit/ml) in SFL3	37	81	
3a	Control in SFL3	0 3	77 74	
3b	Penicillin (1 unit/ml) in SFL3	81 81	93 91	

^a Compared to the viable counts of the bacillary suspensions before lysozyme addition, survival in the different experiments after the 90-min, step 1 incubation was as follows: 1a, 60%; 1b, 60%; 2a, 71%; 2b, 76%; 3a, 44%; 3b, —.

presence of the inhibitor. In this important respect it differs from the recovery shown, for instance, in sample 5 of Table 4, where reversion resumes in the absence of actinomycin D because protein synthesis takes place in the gelatin (see below). One possible explanation of the escape phenomenon is that the inhibitors lose their effectiveness in the gelatin in the course of the incubation. However, this simple explanation is clearly ruled out by the long-term survival data given in Table 6. The fact that the viable count remained about the same in the presence of each of the inhibitors after 17 or 24 hr in GFR medium while extensive multiplication occurred in the antibiotic-free controls indicates clearly that the inhibitors remained effective.

In brief then, the transition from L colony-forming protoplast to bacillary colony-forming walled body does occur in gelatin containing chloramphenicol, puromycin, or actinomycin D. However, although actinomycin D and puromycin do not block the transition, they slow it down markedly; chloramphenicol slows it very slightly.

Inhibition in step 2 by RNA or protein synthesis inhibitors is greatly dependent upon the completeness of priming attained in step 1. This conclusion is supported by experiments such as those shown in Table 7. None of the fully primed samples was inhibited by chloramphenicol, except the 1-hr sample in experiment 3, which showed

TABLE 6. Step 2: effect of chloramphenicol, puromycin, and actinomycin D

Inhibitor in gelatin	Per cent reversion ^a (and survival) in gelatin in										
	Expt 1					Expt 2					
	0 hr	1 hr	2 hr	3 hr	17 hr	0 hr	1 hr	2 hr	3 hr	24 hr	
None	0.1 (82) 0 (74)	26 (58) 20 (74)	25 (49) 29 (56)	69 (53)	100 (13,600) ^b	0 (100) 0.2 (110)	14 (87)	55 (106) 53 (95)	78 (84) 72 (84)	100 (>10 ⁶) ^b	
Chloramphenicol (20 µg/ml) ^c		8 (45) 10 (35)	36 (31) 37 (36)	52 (41)	92 (38)		1 (102)	59 (94) 51 (65)	71 (98) 73 (107)	100 (45)	
Puromycin (10 or 20 µg/ml) ^d		2 (41) 2 (29)	4 (43)	14 (44)	98 (35)		1 (121)	34 (89) 34 (84)	53 (71) 52 (74)	84 (102)	
Actinomycin D (1 µg/ml)		1 (45)	12 (42) 16 (45)	20 (54)	99 (50)		1 (104)	22 (95) 15 (86)	52 (107) 54 (82)	90 (104)	

^a Figures in roman type are reversion percentages, figures in italics and parentheses are the corresponding survival percentages. Each set of figures represents a gelatin tube. Per cent survival was calculated by taking the viable count after protoplasting as 100%. In experiment 1, 59% of the cells survived protoplasting; in experiment 2, 58% survived. Presenting data separately for viability losses during protoplasting and for losses during subsequent step 1 or step 2 incubation permits more informative attribution of these losses to protoplast fragility, imperfect sampling from gelatin tubes, or, possibly, drug action.

^b In the absence of inhibitors, extensive multiplication occurred, starting 4 to 7 hr after inoculation (13).

^c Chloramphenicol concentrations of 32 µg/ml (two experiments) and 100 µg/ml (two experiments) gave results similar to those obtained with 16 to 20 µg/ml.

^d In experiment 1, 10 µg/ml; in experiment 2, 20 µg/ml.

slightly less reversion. However, whenever protein synthesis was restricted in step 1, be it because of the absence of the CH enrichment as in experiment 1 or because of the presence of chloramphenicol or actinomycin D as in experiments 2 or 3, chloramphenicol produced marked inhibition as late as 3 to 4 hr after inoculation into gelatin. Thus, it appears that, if protein synthesis is inhibited before inoculation into gelatin, it must be allowed to occur in the gelatin if reversion is to take place.

Step 2 (reversion): effect of 2,4-dinitrophenol and of hydroxylamine. The inhibitor 2,4-dinitrophenol (14) effectively inhibits reversion when added to gelatin at 3×10^{-3} M. Similarly, hydroxylamine, an inhibitor of macromolecular biosyntheses [including RNA and protein biosynthesis (20)], efficiently blocks reversion at 10^{-3} M. Representative experiments are shown in Table 8.

Step 2 (reversion): effect of the wall synthesis inhibitors penicillin and cycloserine. Since electron-microscopic studies of reversion have already

demonstrated that wall formation is a prominent feature of reversion in gelatin (13) and, since bacilli grow into L colonies in the presence of penicillin, it was expected that penicillin would inhibit reversion in gelatin. This was indeed found (Table 9). Cycloserine, which blocks mucopeptide synthesis at a different point (24), also slowed reversion during step 2. The relatively ineffective inhibition by cycloserine (Table 9) may have been due to the presence of L-alanine in the medium. This amino acid may be racemized to D-alanine, and D-alanine in turn antagonizes the action of cycloserine (24).

Step 2 (reversion): effect of momentary melting of the gelatin. Recently, it was shown that ongoing reversion can be interrupted and partly reversed if the gelatin is momentarily heated at 40 C and then cooled again. This result was attributed to the disturbance of a postulated close juxtaposition of protoplasts and their secreted nascent wall material or wall-forming enzymes during wall initiation (13). It was important to

TABLE 7. Influence of incubation conditions during step 1 on chloramphenicol inhibition during step 2

Expt	Incubation conditions during step 1	Inhibitors in gelatin	Per cent reversion (and survival) ^a in gelatin after	
			Time 1 ^b	Time 2
1a	SFL3	None	61 (55) 96 (104)	99 (144) 99 (95)
1b	SFL3	Chloramphenicol (16 µg/ml)	84 (97) 88 (119)	94 (81) 96 (73)
1c	SFL4	None	1 (111) 2 (98)	44 (68) 23 (74)
1d	SFL4	Chloramphenicol (16 µg/ml)	2 (111) 0 (43)	7 (50) 5 (34)
2a	SFL3	None	88 (82)	97 (103) 96 (59)
2b	SFL3	Chloramphenicol (16 µg/ml)	87 (113)	92 (96) 95 (77)
2c	SFL3 + 16 µg of chloramphenicol per ml	None	2 (116) 5 (63)	26 (62) 31 (46)
2d	SFL 3 + 16 µg of chloramphenicol per ml	Chloramphenicol (16 µg/ml)	2 (88)	4 (50) 8 (70)
3a	SFL3	None	27 (60) 21 (61)	98 (104) 94 (158)
3b	SFL3	Chloramphenicol (16 µg/ml)	16 (77)	96 (107)
3c	SFL 3 + 1 µg of actinomycin D per ml	None	0 (102) 1 (69)	53 (101) 65 (92)
3d	SFL 3 + 1 µg of actinomycin D per ml	Chloramphenicol (16 µg/ml)	0 (93)	0.4 (170)

^a Figures in roman type are reversion percentages. Figures in parentheses and italics refer to survival in the gelatin after incubation. Survival of protoplast suspensions at time 0 relative to parent bacillary suspensions was as follows: experiments 1a, 1b: 63%; 1c, 1d: 74%; 2a, 2b: 81%; 2c, 2d: 86%; 3a to d: 48%.

^b For experiment 1 (a to d): time 1, 2 hr; time 2, 4 hr. For experiment 2 (a to d): time 1, 2.5 hr; time 2, 3.5 hr. For experiment 3 (a to d): time 1, 1 hr; time 2, 3 hr.

TABLE 8. Step 2: inhibition by 2,4-dinitrophenol and by hydroxylamine

Expt	Inhibitor in gelatin	Per cent reversion (and survival) ^a in gelatin after		
		1 hr	2 hr	3 hr
1a	None		43 (75) 47 (76)	
1b	Dinitrophenol (3×10^{-3} M)		2 (85) 2 (88)	
2a	None	47 (83) 16 (102)		77 (105) 92 (179)
2b	Hydroxylamine (10^{-3} M)	0.3 (162) 0 (113)		0.2 (98) 24 (113)

^a Figures in roman type are reversion percentages. Figures in parentheses and italics refer to survival in the gelatin after incubation; survival in the zero-time sample is taken as 100%. Survival in the zero-time samples relative to the parent bacillary suspensions was as follows: experiments 1a, 1b: 75%; 2a: 34%; 2b: 43%.

TABLE 9. Step 2: effect of the wall synthesis inhibitors penicillin and cycloserine

Expt	Inhibitor in gelatin	Per cent reversion (and survival) in gelatin after			
		1 hr	2 hr	3 hr	24 hr
1 ^a	None	14 (87)	55 (106) 53 (95)	78 (84) 72 (84)	100 (>10 ^b)
	Penicillin G (1 unit/ml)	0 (126)	0 (99) 0 (104)	0.3 (52) 0 (69)	3 (53) ^b
2 ^b	None	40 (93)	80 (116) 76 (67)		
	Cycloserine (100 μ g/ml)	20 (78)	35 (85)		
	Cycloserine (400 μ g/ml)	2 (53)	34 (60)		

^a This experiment formed part of experiment 2, Table 6; control data and basis for calculating survival are the same.

^b Of the population, 97% formed L colonies.

^c As in the penicillin experiment, per cent survival was calculated by taking survival after protoplasting as 100%; 62% of the cells survived protoplasting.

repeat this experiment with protoplasts preincubated in high-CH medium to ascertain whether the heat-arrest of reversion could be associated with step 2 of reversion. The heat effect was indeed demonstrable with primed protoplasts, although it was less clear-cut than in the slower, unprimed system (Table 10). As one might expect, the moment when heating is most effective, namely at 0.5 hr after transfer to gelatin, was earlier in the present system than in the preceding experiments, in which the most effective time was 4 hr after inoculation (13). In the earlier experiments, protein synthesis in the gelatin had to prime the protoplasts first, presumably before they could attain the disturbance-sensitive stage. In separate experiments, with protoplasts preincubating in CH, we also ascertained that one or more heat treatments during preincubation

has no influence on the reversion rate in gelatin (data not shown).

Step 3: osmotic resistance of cells late in reversion. In studies concerned with successive steps in protoplasting, an osmotically sensitive, transient rod stage was found to appear early in lysozyme treatment. During this stage, cells gave rise to bacillary colonies on such media as SDG and DP but could not grow on media lacking osmotic stabilizer (12, 17). In search of a similar stage during reversion, reverting cells were often plated on unstabilized media such as S⁻, Blood Agar Base, or Brain Heart Infusion after resuspension from gelatin incubation, and the count was usually lower on the hypotonic media. This lag in the acquisition of osmotic resistance was greatly accentuated by chloramphenicol, puromycin, and actinomycin D (Table 11). Experi-

TABLE 10. Interruption of reversion by brief heat treatments

Time after inoculation into gelatin		Per cent reversion (and survival) ^b in gelatin
When heated ^a	When assayed for reversion	
<i>hr</i>	<i>hr</i>	
—	1	44 (105) 44 (82)
0.5	1	22 (88) 21 (109)
—	2	91 (104) 89 (97)
0.5	2	80 (101)
0.5 + 1	2	65 (79)
1	2	63 (63) 60 (97)
1 + 1.5	2	68 (90) 68 (95)

^a Heating was by immersion of the gelatin tubes in a waterbath at 40 C for 3.5 min without agitation. Immediately thereafter, the tubes were returned to the waterbath at 26 C.

^b Figures in parentheses and italics refer to survival in the gelatin after incubation, with survival at zero time taken as 100%. Survival of protoplasts at zero time relative to parent bacillary population was 28%.

ments 1 and 2 (Table 11) show that, after 4 hr in gelatin, the revertant bacilli acquired their ultimate osmotic resistance, and about 80% of them survived plating on hypotonic medium (compare experiment 3a). By contrast, when chloramphenicol, puromycin, or actinomycin D had been present in the gelatin since zero time, only a small percentage of the revertant bacilli became osmotically resistant. The block by chloramphenicol and by actinomycin D was very effective and persisted for 17 to 24 hr. However, puromycin blocked only partially, and, in the two late samplings, 3c and 4c (Table 11), respectively, 58 and 57% of the revertants became osmotically stabilized.

DNA synthesis and reversion. In view of the involvement of protein synthesis in various stages of reversion and in view of much current speculation concerning the coordination of chromosome synthesis with cell division and, hence, crosswall formation (2, 4, 9, 11, 15), it was pertinent to inquire about the role of DNA synthesis in reversion.

For these experiments we employed strain SB566, a thymine- and tryptophan-requiring strain.

In the experiment shown in Table 12, the chromosomes of the inoculum bacillary popula-

TABLE 11. Step 3: development of osmotic resistance

Expt	Reagent in gelatin	Incubation in gelatin	Avg bacillary count	Avg bacillary count	B/A × 100 ^b
			on osmotically stabilized medium ^a A	on hypotonic medium ^a B	
		<i>hr</i>			
1a	None	4	224 257	208 200	93 78
1b	Chloramphenicol (16 μg/ml)	4	239 125	37 18	15 14
2a	None	4	436	354	81
2b	Puromycin (12 μg/ml)	4	237	12	5
2c	Actinomycin D (1 μg/ml)	4	299	10	3
3a	None	17	34,900	27,500	79
3b ^c	Chloramphenicol (20 μg/ml)	17	90	12	13
3c ^c	Puromycin (10 μg/ml)	17	88	51	58
3d	Actinomycin D (1 μg/ml)	17	126	24	19
4a	None	24	>10 ⁶	>10 ⁵	
4b ^c	Chloramphenicol (20 μg/ml)	24	88	4	5
4c ^c	Puromycin (20 μg/ml)	24	167	95	57
4d	Actinomycin D (1 μg/ml)	24	204	3	1

^a Averaged plate counts are given directly except in the case of tubes 3a and 4a; here multiplication occurred and the actual counts were obtained from 100-fold higher dilutions.

^b Per cent bacilli which were osmotically resistant.

^c This experiment is also cited in Table 6.

TABLE 12. Effect of thymine starvation on reversion

Thymine in preincubation	Per cent reversion (and survival) ^a in gelatin after					
	2 hr		4 hr		15 hr	
	With thymine	Without thymine	With thymine	Without thymine	With thymine	Without thymine
No	33 (99)	45 (53) 55 (83)	74 (71)	84 (71) 88 (31)	100 (13,200)	100 (5)
Yes	42 (95)	22 (82) 41 (79)	91 (95)	74 (68) 83 (47)	100 (17,200)	100 (14)

^a Figures in roman type are reversion percentages; figures in parentheses and italics refer to survival in the gelatin tubes after incubation; survival at zero time is taken as 100%.

tion were aligned at the terminus by a 4.5-hr period of incubation with thymine in the absence of tryptophan and amino acids (2). After two washings in the thymine-free SFL5 medium, the cells were frozen. Upon melting, they were again washed in SFL5 and incubated for 30 min at 37 C to eliminate all traces of thymine. Then the culture was diluted into thymine-free SFL5 or SFL5 supplemented with 20 μ g of thymine per ml, respectively. Both subcultures were protoplasted for 90 min; survival was 43% in the thymine-free culture and 36% in the thymine-supplemented one. Dilutions from both suspensions were inoculated into thymine-free gelatin tubes and thymine-supplemented gelatin. Sample tubes were withdrawn, melted, diluted, and plated at 2, 4, and 15 hr. Table 12 shows that, regardless of whether thymine was present or absent during step 1, step 2, or both, reversion proceeded at about the same rate. Clearly, chromosome initiation or synthesis is not required for reversion, nor is reversion promoted by the presence of thymine. The same result was also obtained in two experiments in which the effect of thymine deprivation during steps 1 and 2 was studied in media containing different CH supplements. The fact that thymine starvation was indeed effective is indicated by the 15-hr viability figures (Table 12) which show thymineless death in the thymine-free samples and considerable multiplication in the thymine-supplemented tubes. The low incidence of thymineless death in the 2- and 4-hr samples is consistent with the earlier finding that thymineless death is absent or very slow in osmotically stabilized cultures (2).

Priming L bodies for reversion. In almost all of the preceding experiments, freshly prepared protoplasts were used as inoculum because protoplast inocula of uniform morphology, of predictable viability, and with very low bacillary contamination can be obtained conveniently. In earlier

experiments, resuspended L colonies were often used as inocula. However, when these two types of wall-less cells were compared, they always exhibited essentially the same reversion behavior (12, 13). In the current experimental series, a spot check comparison was also made (Table 13). An L-body suspension was prepared as described above. After melting, the suspension was divided and the two subcultures were incubated for 70 min at 33 C in SFL3 and SFL4 media, respectively. Dilutions of the two suspensions were then inoculated into gelatin tubes for a routine reversion test. Table 13 shows that L bodies, like protoplasts, can be stimulated to revert very rapidly in gelatin by prior incubation in high-CH medium. As in the case of protoplasts, chloramphenicol in step 2 had no effect on the reversion of cells preincubated in SFL3.

DISCUSSION

What is reversion? We have previously stressed the point that *B. subtilis* protoplasts are entirely devoid of cell wall and that they and their progeny continue in the wall-less state indefinitely (propagating as L-forms), unless and until a special priming process restarts the normal self-sustaining (feedback) mechanism for wall biosynthesis (11, 12, 13, 22). As the cell wall is removed, loss of mesosomes and loss of the ability of the cells to form septa ensues (22). Both return to normal only after wall formation, and often mesosomes are not formed for several cell generations after resumption of normal division (13). In studying reversion, one is thus examining, for the most part, the priming and the early functioning of cell wall biosynthesis. Quite likely, some of the processes important in priming also play a role in maintaining cell wall biosynthesis in normally growing cells.

Complexity of reversion. A simple view concerning this system is that a particular element in the

TABLE 13. *Priming L bodies for reversion*

Incubation conditions during step 1	Inhibitor in gelatin	Per cent reversion (and survival) ^a in gelatin after	
		1 hr	2 hr
SFL3	None	47 (78)	80 (111)
		68 (78)	82 (108)
SFL3	Chloramphenicol (16 µg/ml)		82 (109)
SFL4	None	11 (140)	45 (108)
		6 (79)	62 (90)

^a Figures in roman type are reversion percentages. Figures in parentheses and italics refer to survival in the gelatin after incubation; survival in the L body suspension before step 1 preincubation is taken as 100%.

wall-forming feedback chain is lost as a result of wall removal and that replacement of this element during reversion permits resumption of wall biosynthesis (22). The present research and other recent results show that such a picture is too simple and that, in fact, a series of successive, biochemically complex phases must be traversed during reversion (Fig. 1).

Step 1. During the phase labeled "step 1," processes occur which entail a prolonged period of protein and RNA synthesis. Our observations that these macromolecule biosyntheses require so much time (Table 1) and that they must occur while the cell is naked (Table 3) suggest that these are not ordinary enzyme biosyntheses. Perhaps a modification of the cell membrane is taking place. However, no obvious changes in the protoplast membrane are evident in the electron microscope at the end of a 90-min incubation period in SFL3 (A. Ryter, *personal communication*). Several observations indicate that mucopeptide wall synthesis is not taking place during this phase: penicillin does not inhibit (Table 5); lysozyme has no effect; and diaminopimelic acid (DAP) biosynthesis, which is promptly and completely repressed upon wall removal, remains repressed (E. Bond, Ph.D. Thesis Georgetown Univ., Washington, D.C., 1969). No wall is visible in the electron microscope.

Step 2. By contrast, all of the available evidence indicates that mucopeptide synthesis does take place during the second step of reversion: penicillin and cycloserine inhibit this step (Table 9), lysozyme quickly reverses incipient reversion, and wall appears in the electron microscope (13). Apparently, derepression of DAP biosynthesis occurs in the gelatin and other reversion environments; however, the mechanism initiating this derepression is not known (E. Bond, Ph.D. Thesis, Georgetown Univ., Washington, D.C., 1969). Some facets of the events of step 2, and perhaps DAP derepression is included in these, require that the cell be in close contact with a

solid substratum (here provided by gelatin, elsewhere by hard agar, membrane filters, cell wall fragments, or autoclaved microorganisms (11, 12; D. Clive, Ph.D. Thesis, Georgetown Univ., Washington, D.C., 1968). Even a momentary loss of this solid substratum, engendered by melting of the gelatin, produces a marked inhibitory effect on reversion (Table 10). We have interpreted this sensitivity to physical disturbance to indicate that, during early stages of wall formation in gelatin, localized extracellular accumulations of wall polymers or wall-synthesizing enzymes occur which are not well affixed to the protoplast surface and are swept away by fluid motion during melting (13).

In view of the large biosynthetic task of wall building during step 2, the requirement for metabolic energy, as revealed by sensitivity to 2,4-dinitrophenol, was fully expected. By contrast, the marked transient sensitivity of this step to actinomycin D and puromycin and its virtual lack of sensitivity to chloramphenicol are quite puzzling. Taking the results at their face value, a process sensitive to actinomycin D and puromycin (and relatively insensitive to chloramphenicol) is rate-limiting in the gelatin phase of reversion, yet this process can go to completion even in the presence of the inhibitors. Conceivably, a partial requirement for a special soluble RNA involved in cell-wall biosynthesis (3) could explain these results.

Step 3. The discovery that cells pass through an osmotically sensitive bacillary phase during reversion is not surprising. It seems quite plausible that bacilli would remain osmotically sensitive until a wall of near normal thickness is built. It is surprising, however, that the development of osmotic resistance is blocked by chloramphenicol (Table 11), whereas the initial phases of wall formation are quite insensitive to this antibiotic (Tables 6 and 7). Since chloramphenicol does not prevent mucopeptide biosynthesis (6) and the formation of extrathick walls is a feature asso-

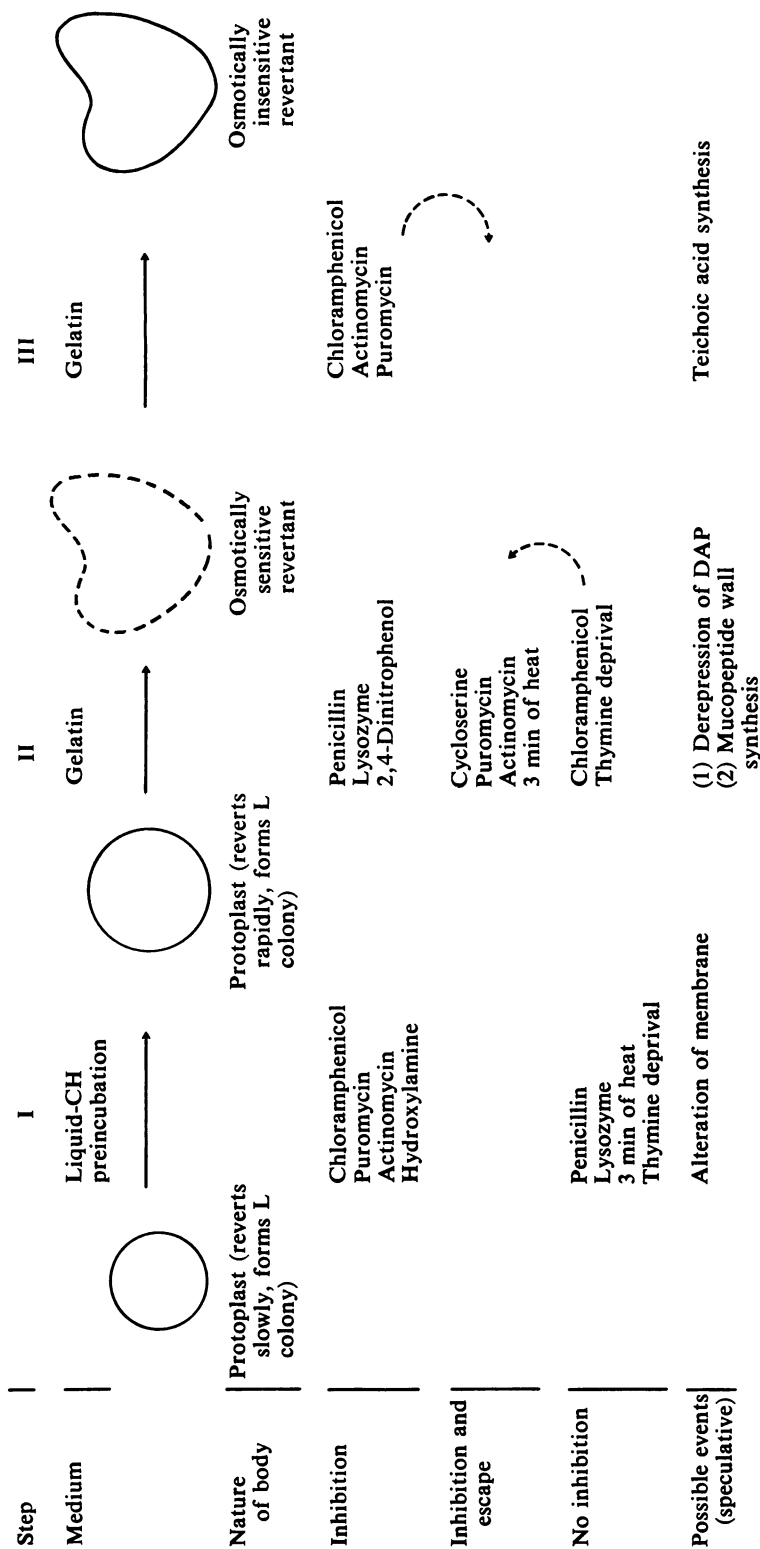


FIG. 1. Phases of the reversion process.

ciated with incubation in chloramphenicol media (17) or, more generally, with incubation under conditions of protein synthesis inhibition (7, 23), it appears that mere accretion of mucopeptide is insufficient to render cells osmotically resistant. A reaction requiring RNA and protein synthesis seems to be necessary. We can only guess what this reaction may be. One such guess is that the reaction in question is the synthesis of teichoic acid: incorporation of ^{32}P into teichoic acid is blocked by chloramphenicol (2).

DNA synthesis and initiation of wall biosynthesis. When a chromosome replication cycle has been completed, the membrane region between the attachment sites of the newly completed chromosomes is thought to begin localized growth, separating the sister chromosomes (21). Later a double membrane septum arises by centripetal growth between the two attachment sites, accompanied by cell wall secretion into the space between the two septal membranes. In this indirect manner, wall synthesis is coordinated with chromosome synthesis. If chromosome synthesis or an open chromosome replication fork were required for initiation of wall synthesis, thymine-starving cells with chromosomes arrested at the terminus should not be able to revert to the bacillary form. In fact, reversion occurs normally in such cells (Table 12). We conclude that coordination between DNA synthesis and cell wall synthesis is indirect. This inference is, of course, consonant with the finding that DNA replication continues unrestricted in the complete absence of cell wall in L-forms, and with the finding that wall synthesis and cell separation can occur without concomitant chromosome synthesis in mutants (8) and other systems (2).

Reversion and development. In an earlier paper, we pointed to the parallelism between reversion and morphogenesis in higher organisms (11). This comparison was based on the fact that in both systems, in the face of an unchanging genic constitution, a morphological phenotype which persists through many cell generations may suddenly be changed into a new heritably persistent phenotype in response to a stimulus at the cell surface. Details of reversion revealed by the present study further underline this analogy. It is now clear that, far from being a simple, single event, reversion requires successive, coordinated phases of macromolecule biosynthesis. Furthermore, at a certain stage, a physically rigid environment in contact with the cell surface (requirement for chemical characteristics are not specific) must somehow trigger derepression of previously repressed wall precursor biosynthesis (E. Bond, Ph.D. Thesis, Georgetown Univ., Washington, D.C., 1969). These parallelisms suggest that, in

reversion, bacterial protoplasts may already employ induction mechanisms, similar to those which assume key importance in development at higher evolutionary stages.

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