JOURNAL OF BACTERIOLOGY, May 1983, p. 916–923
0021-9193/83/050916-08$02.00/0

Vol. 154, No. 2

Streptococcus faecium ATCC 9790 Penicillin-Binding Proteins and Penicillin Sensitivity Are Heavily Influenced by Growth Conditions: Proposal for an Indirect Mechanism of Growth Inhibition by β-Lactams

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Received 26 October 1982/Accepted 26 February 1983

The effects of variations in growth conditions on the penicillin response of *Streptococcus faecium* ATCC 9790 were studied. Changes in the growth temperature and medium composition were found to cause striking changes in the bacterial generation time, cellular penicillin sensitivity (minimum inhibitory concentration), sensitivity of peptidoglycan synthesis to inhibition by penicillin, rate of autolysis, and labeling pattern of penicillin-binding proteins. However, no constant relationship between these parameters and the minimum inhibitory concentration could be observed. Similar electrophoretic patterns for penicillin-binding proteins were observed in cells grown in different media at the optimal growth temperature. Inhibition of cell division by penicillin in cells grown at this temperature (but not at higher or lower temperatures) caused filamentation of the bacteria. In cells grown in a chemically defined medium at the optimal temperature (but not at temperatures above or below), complete inhibition of cell division was associated with only partial inhibition (34% after 150 min) of peptidoglycan synthesis. It is suggested that the status and physiological importance of individual penicillin-binding proteins in *S. faecium* are heavily influenced by growth conditions. Depending on the growth conditions, different penicillin-binding proteins may perform the cellular function, indispensible for bacterial growth.

The first mechanism suggested to explain the growth-inhibitory effect of penicillin upon susceptible bacteria was inhibition of transpeptidase reaction and prevention of cross-linkages in newly formed peptidoglycan of *Staphylococcus aureus* (20, 23). However, more recent studies with other microorganisms have pointed to a variety of other biochemical effects of penicillin that could also be important in the mechanism of the antibacterial effects (7–10, 12, 13). It is also known that structurally different β-lactams may cause antibacterial effects quite different from those caused by penicillin (8, 11, 12, 14).

Some of this variation in mode of action is most likely related to the fact that the β-lactam antibiotics recognize multiple targets (penicillin-binding proteins [PBPs]) in bacterial cells (1, 19). To explain the connection between these multiple targets and the variety of growth-inhibitory effects of β-lactam antibiotics, the role that PBPs play in cellular physiology and the mode in which they take part in essential physiological functions should first be elucidated.

A sensitive dependence of the inhibitory activity on the growth rate of the target bacteria is a peculiarity of β-lactam antibiotics. We have recently shown that *Streptococcus faecium* grown in chemically defined medium (CDM) shows different PBP labeling patterns at different temperatures and that in cells growing in this medium at the fastest growth rate the target most likely to be responsible for growth inhibition by penicillin may be PBP 3 (6).

In this study we have analyzed the effect of various growth conditions on the growth rate, susceptibility to penicillin, PBP labeling, and some other relevant physiological parameters of *S. faecium*. Although all these functions showed variations dependent on growth conditions, no constant relation could be established between susceptibility to penicillin and any of the other physiological parameters tested. We conclude that growth inhibition by β-lactam antibiotics is caused by an indirect mechanism.

MATERIALS AND METHODS

Organism and growth conditions. *S. faecium* ATCC 9790 was used in all experiments. Cells were grown in

the CDM described by Shockman (17), in streptococcal broth (SB, see below), and in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Michigan). SB contained (per liter of distilled water) 10 g of yeast extract, 10 g of Bacto-Peptone (Difco), 0.442 g of KH_2PO_4, 0.305 g of K_2HPO_4, 25.65 g of Na_2HPO_4, 16.45 g of NaH_2PO_4, and 20 g of glucose. Cultures were incubated in a New Brunswick water bath, model G86 (New Brunswick Scientific Co., New Brunswick, N.J.) with temperature control within ±0.25°C. Before experiments, cells were subcultured under the desired conditions three times. Growth was monitored with a Perkin-Elmer spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.).

Susceptibility tests and morphology studies. The broth dilution test was used to determine the minimum inhibitory concentration (MIC) of penicillin for *S. faecium*. Twofold dilutions of the antibiotic in CDM, SB, and BHI broth were inoculated with stationary-phase cells to give a final density of 10^5 cells per ml, and the cells were then incubated at the desired temperature. The MIC was considered to be the lowest concentration of penicillin that prevented turbidity after 18 h of incubation (22).

To evaluate the effect of penicillin on cell division, the antibiotic was added to an exponentially growing cell culture (10^7 cells per ml) to obtain the desired final concentration. At intervals, samples were taken, and cells were counted with a Coulter Counter model D (Coulter Electronics, Inc., Hialeah, Fla.). The lowest dose which inhibited an increase in the number of cells after 3 h of incubation was called the minimum division-inhibiting concentration (MDC). Other samples, taken at the same intervals, were used to examine bacterial morphology with an interference or a phase-contrast microscope.

Cellular autolysis. Exponentially growing cells (10 ml) were collected by filtration, washed with ice-cold distilled water, and suspended in 0.3 M phosphate buffer (pH 7) (3) to give an initial turbidity of 1 optical density unit. Tubes were incubated at the same temperature employed for growth, and lysis was monitored turbidimetrically. The lag before autolysis was short (not exceeding 5 min) under all conditions. To compare the autolysis of different samples, results are expressed in units of autolysis, one unit being equivalent to a loss of 1% of the initial turbidity per hour (15).

Peptidoglycan synthesis. Peptidoglycan synthesis was measured by the incorporation of [^{3}H]lysine in the pronase-resistant cell fraction as described by Boothby et al. (2). Cultures were grown for at least eight generations in CDM containing [^{3}H]lysine (2 μCi; specific activity, 25 Ci/mmol; Radiochemical Centre, Amersham, England) and 20 μg of cold lysine per ml or [^{3}H]leucine (0.5 μCi/ml; specific activity, 50 Ci/mmol; Radiochemical Centre) and 20 μg of cold leucine per ml. The latter sample was used to control the efficiency of pronase in removing proteins from the peptidoglycan fraction. The radioactive cultures were then diluted with the same fresh medium to a concentration of 10^7 bacteria per ml and were exposed to various concentrations of penicillin. Twin samples (0.5 ml) from antibiotic-treated and untreated cultures were taken at intervals and precipitated with trichloroacetic acid (final concentration, 10%). The acid precipitates were hydrolyzed at 95°C for 15 min, collected on glass fiber (Whatman GF/C; Whatman, Inc., Clifton,

TABLE 1. Effects of temperature and growth medium on *S. faecium*

Temp (°C)	Generation time (min)			Maximum growth[a]			Benzylpenicillin MIC (μg/ml)			Autolysis[b]		
	CDM	SB	BHI	CDM	SB	BHI	CDM	SB	BHI	CDM	SB	BHI
30	50	40	46	1.4	1.4	1.2	8	8	2	10	14	24
32	47	38	41	1.4	1.4	1.2	4	2	0.25	12	20	45
37	45	26	40	1.4	1.4	1.2	4	0.25	0.25	18	45	64
42	32	29	38	1.4	1.2	1.2	0.5	0.016	0.016	24	58	75
45	31	41	43	1.4	0.8	0.6	0.04	0.004	0.004	56	85	87
47	45	67	83	0.8	0.8	0.4	0.02	0.004	0.004	80	26	35
49	120	NG[c]	NG	0.5	NG	NG	0.02	ND[d]	ND	27	ND	ND

[a] Maximum growth was measured during the early stationary phase of growth and is expressed as optical density units.
[b] One lysis unit is equivalent to a loss of 1% of initial turbidity per hour (15).
[c] NG, No growth.
[d] ND, Not determined.

TABLE 2. Effects of temperature and growth medium on susceptibility of *S. faecium* to rifampin, chloramphenicol, and vancomycin

Temp (°C)	MIC (μg/ml)								
	Rifampin			Chloramphenicol			Vancomycin		
	CDM	SB	BHI	CDM	SB	BHI	CDM	SB	BHI
32	1.2	2.5	5	2.5	0.3	2.5	0.15	0.3	0.3
45	1.2	2.5	5	2.5	0.3	2.5	0.3	0.3	0.6

N.J.), and washed twice with 5 ml of ice-cold 10% trichloroacetic acid, twice with 2 ml of 95% ethanol, and once with 2 ml of 0.05 M Tris, pH 7.8. One of the two filters was then placed in a vial and treated with 2 ml of pronase (1 mg/ml) in 0.05 M Tris, pH 7.8, for 30 min at 37°C. After incubation, the filters were washed with 4 ml of distilled water and then with 4 ml of absolute ethanol. They were next air dried and transferred to scintillation vials. Filters were then treated with 90% NCS, and radioactivity was counted in a Packard liquid scintillation counter (Packard Instrument Co., Inc., Rockville, Md.). The evaluation of the radioactivity remaining on the filter before and after pronase digestion of both [^{3}H]lysine and [^{3}H]leucine samples indicated that this method resulted in a peptidoglycan fraction that was contaminated with 5 to 8% protein only. The total amount of [^{3}H]lysine radioactivity incorporated by cells was 1.6×10^3 cpm/μg (dry weight) under all the growth conditions.

Detection of PBPs. Membranes were prepared from cells growing exponentially under various conditions as described previously (4, 5). Samples (20 μl) containing 150 μg of protein were treated with [^{14}C]benzylpenicillin (specific activity, 59 Ci/mol; Radiochemical Centre) at a final concentration of 100 μM for 15 min at the desired temperature followed by the addition of nonradioactive benzylpenicillin (final concentration, 100 mM) and 20 μl of buffer containing sodium dodecyl sulfate. The resulting solution was then submitted to polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate, and the PBPs were detected by fluorography as described (4, 5). The amount of radioactivity bound to PBPs was estimated by microdensitometry of the fluorograms with a Joyce Loebl MK3CS densitometer. To evaluate the exposure time required to obtain image densities of PBPs that were sufficient for accurate densitometry, the gels were exposed to X-ray film for different lengths of time. It was found that 20 days of exposure allowed a linear film response for the majority of PBPs under all the growth conditions. In certain samples, a linear film response for PBP 6 was obtained within a time length which was insufficient to detect some PBPs. For this reason, PBP 6 in some samples was underestimated.

To ensure correct comparison of patterns, all the gels were exposed to X-ray film at the same time. Thus, the time of exposure was exactly the same for all samples, and variations in darkroom procedure were avoided.

RESULTS

Effect of temperature and medium composition on growth rate, susceptibility to penicillin, and autolytic activity. Table 1 lists generation times together with the maximum growth yields, penicillin MICs, and the rates of autolysis of *S. faecium* grown at various temperatures in three different media. Both the medium and the growth temperature heavily influenced the generation time. In SB only, the shortest generation time was observed at 37°C. In BHI broth and CDM, temperatures optimal for growth were 42 and 45°C, respectively. Medium composition and temperature had less drastic effects on growth yield. Rates of autolysis of bacteria suspended in buffer differed in the three media, and in all of them the rate increased with temperature up to the second highest temperature tested and then drastically dropped. Susceptibility to penicillin (measured by the MIC) was almost equal in SB and BHI broth but was much different in CDM. In all three media, it was found to increase with growth temperature up to the second highest temperature tested, and then it remained constant. Thus, the MIC did not show parallel variation with any of the parameters tested.

Variations in medium composition and growth temperature had only minor effects on the susceptibility of cells to vancomycin, another inhibitor of cell wall synthesis, and had virtually no effect on the MICs of rifampin and chloramphenicol, which inhibit RNA and protein synthesis, respectively (Table 2).

Analysis of the PBPs in the membranes of *S. faecium* cells grown in different media at different temperatures. The status of PBPs was dramatically influenced by the growth medium (Fig. 1, Table 3). In SB, the image density of PBPs either increased slightly (PBPs 1, 2, and 4) or remained virtually unvaried (PBPs 3 and 6) between 30 and 37°C; it gradually decreased in the cells grown at temperatures above 37°C. In contrast, in CDM the image density of PBPs remained the same between 30 and 37°C, gradually increased from 37 to 45°C, and decreased at higher temperatures. The exception was PBP 6, whose image density slightly decreased at temperatures above 37°C. In BHI broth, the image density of PBP generally increased with temperature up to 42°C and then decreased. The exceptions were PBP 6, whose image density slightly decreased, and PBPs 3 and 4, whose image density did not vary significantly between 30 and 37°C.

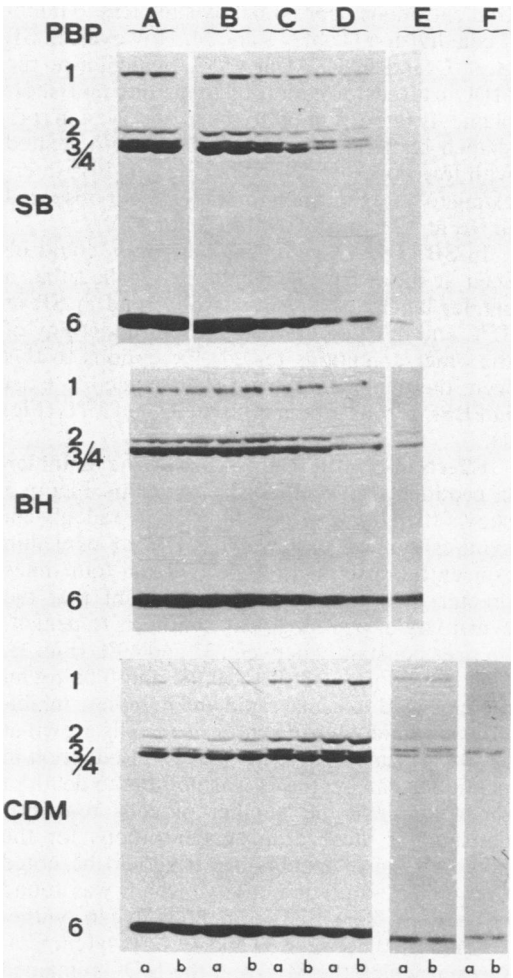

FIG. 1. PBP of *S. faecium* grown under different conditions. Membranes were prepared from cells exponentially growing in CDM, BHI broth, and SB at temperatures of 32 (A), 37 (B), 42 (C), 45 (D), 47 (E), and 49°C (F). Two samples (150 μg of protein) of each membrane preparation were labeled with 100 μM [^{14}C]benzylpenicillin for 15 min, one at 20°C (a) and the other at the same temperature used for growth (b). The proteins were then fractioned by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the PBPs were detected by fluorography. Binding of membranes from cells grown in BHI broth and SB at 47°C was performed only at 47°C. PBP 5 was not detected on these gels.

The values reported in Table 3 were obtained under conditions of linear film response for all the PBPs except PBP 6. Under certain growth conditions, the amount of PBP 6 was so high that accurate measurement was only possible after a time too short to detect the other PBPs (for instance, PBPs 1, 2, and 3 in cells grown at 30, 32, and 37°C in CDM). For this reason, the

TABLE 3. Absolute amount of penicillin bound by each PBP in membranes from *S. faecium* grown under different conditions[a]

Temp (°C)	Area of the peak (mm^2) corresponding to PBP:																					
	1			2			3			4			6									
	CDM	SB	BHI	CDM	SB	BHI	CDM	SB	BHI	CDM	SB	BHI	CDM	SB	BHI	CDM	SB	BHI	CDM	SB	BHI	
30	6 ± 0.2	39 ± 1.5	38 ± 0.5	10 ± 1.6	35 ± 1.5	47 ± 1	12 ± 0.6	120 ± 1.2	110 ± 2.6	100 ± 1	210 ± 1.9	106 ± 1.1	305 ± 1.6	422 ± 2.2	357 ± 0.7							
32	6 ± 0.1	47 ± 1.2	41 ± 0.2	12 ± 0.2	38 ± 3.1	46 ± 0.6	15 ± 0.4	121 ± 1.4	119 ± 0.8	110 ± 1.1	215 ± 2	115 ± 1.6	297 ± 1.1	421 ± 0.2	358 ± 1.7							
37	7 ± 0.1	61 ± 2.7	42 ± 0.2	18 ± 1.6	52 ± 3	56 ± 1.4	15 ± 0.1	125 ± 1.8	115 ± 0.4	112 ± 0.8	250 ± 1.5	120 ± 1	296 ± 1	426 ± 1.4	358 ± 2.4							
42	18 ± 0.3	28 ± 1.5	66 ± 1	37 ± 3.8	13 ± 1.2	72 ± 1.4	67 ± 2.4	76 ± 0.6	117 ± 0.5	113 ± 0.6	108 ± 0.8	119 ± 1.1	230 ± 1.2	210 ± 2	339 ± 1.6							
45	45 ± 1.5	12 ± 0.4	44 ± 1.6	86 ± 2.6	8 ± 0.6	42 ± 1.5	89 ± 2	16 ± 0.7	57 ± 0.5	122 ± 1	25 ± 1	100 ± 1.2	210 ± 1.7	84 ± 0.7	224 ± 1.5							
47	11 ± 0.4	0	0	7 ± 0.9	0	12 ± 0.6	6 ± 0.8	ND	14 ± 0.6	11 ± 1.6	ND	20 ± 0.6	86 ± 1.4	59 ± 1.1	198 ± 2.3							
49	0	ND[b]	ND	0	ND	ND	6 ± 0.8	ND	ND	11 ± 1.6	ND	ND	56 ± 1.2	ND	ND							

[a] The amount of penicillin bound by each PBP was determined by scanning three different fluorograms prepared under identical conditions with a Joyce-Loebl microdensitometer and calculating the areas of the peaks thus obtained. Values, which represent the average of the three different determinations, were approximated to the nearest whole number above or below.

[b] ND, Not determined.

TABLE 4. Radioactivity bound to PBPs in membranes from *S. faecium* grown under different conditions

Temp (°C)	Total radioactivity[a]		
	CDM	SB	BHI
30	433 ± 1	816 ± 6.9	658 ± 6.9
32	440 ± 0.8	842 ± 6.3	679 ± 3.1
37	448 ± 0.8	914 ± 4.9	691 ± 4.9
42	465 ± 7.1	435 ± 1.5	713 ± 2
45	552 ± 8.3	145 ± 0.3	467 ± 4.6
47	140 ± 2.3	59 ± 1.1	244 ± 2.8
49	73 ± 5.3	ND[b]	ND

[a] Values were obtained by adding up the average amount of radioactivity bound to a single PBP, determined as described in Table 3, footnote *a*.
[b] ND, Not determined.

amounts of PBP 6 in cells grown at 30, 32, 37, and 42°C in CDM, BHI broth, and SB reported in Table 3 were underestimated. Nevertheless, this inaccuracy in the measurement of PBP 6 did not significantly affect the interpretation of the results described above.

The total amount of radioactivity bound by PBPs was also influenced by the temperature and the growth medium (Table 4). In each medium, the total radioactivity increased with temperature up to the temperature optimal for growth in that specific medium. At higher temperatures, the bound radioactivity decreased rapidly. In spite of this, the susceptibility of cells to penicillin continued to rise with temperature (Table 1).

To determine whether the different labeling patterns of PBPs at different temperatures could be due to interference with the capacity of PBPs to bind penicillin, membranes were bound in the standard experiments both at the growth temperature and at 20°C. In all media and at all growth temperatures, the electrophoretic patterns of PBPs appeared identical for membranes bound at both temperatures (Fig. 1). Finally, it should be noted that the PBP labeling patterns of membranes from cells grown in the three different media at the respective optimal growth temperatures were very similar.

Effects of penicillin on cell morphology in bacteria grown under different conditions. PBPs are thought to play important roles in septum formation and cell shape regulation. If this is true, conditions that cause changes in PBPs should cause changes in the effects of penicillin on cell shape.

The effects of a wide range of penicillin concentrations on the morphology of cells grown in SB and CDM at different temperatures were examined. Under most conditions, the only observable effect was the formation of ovoid or lemon-shaped cells or chains of coccoid cells at concentrations approximately sufficient to inhibit cell division (Tables 1 and 5). However, in SB at 37°C and in CDM at 45°C, penicillin at the MDC caused formation of long rods and short filaments rather similar to those observed in rod-shaped bacteria (e.g., *Escherichia coli*) treated with low doses of penicillin (Fig. 2). It is interesting to note that such forms were not observed in SB at 45°C or in CDM at 30 or 47°C.

In SB at 32°C, rods and short rods could be seen at the MDC. It should be recalled that a similar labeling pattern was observed in SB at 37°C and in CDM at 45°C, but not under any of the other conditions tested. Exceptions to this were the minor differences that seemed to exist in PBPs of cells grown in SB at 32 and 37°C (Fig. 1).

Effects of growth temperature on the inhibition of peptidoglycan synthesis by penicillin. Figure 3 shows the effects of penicillin on peptidoglycan synthesis in cells grown in CDM at penicillin concentrations equal to and two and four times greater than the MDC. It is evident that the sensitivity of peptidoglycan synthesis to penicillin rises constantly between 32 and 49°C (Fig. 3). Concentrations of 8, 0.16, 0.08, and 0.02 μg/ml were needed to cause rapid and complete inhibition of peptidoglycan synthesis in cells grown at 32, 45, 47, and 49°C, respectively. Reduction in peptidoglycan synthesis was not due to death of the cells since the number of cells remained constant at these drug concentrations for the first 150 min of incubation. It should be noted that unlike autolytic activity, which was found to decrease, sensitivity of peptidoglycan synthesis increased between 47 and 49°C, two temperatures at which the MIC and the MDC remained constant.

An interesting observation may be made by comparing data shown in Fig. 3 and Table 5. It is evident from such data that the MDC of penicillin caused only a 36% inhibition of peptidoglycan synthesis after 150 min of incubation at 45°C, whereas the same concentration caused a drastic inhibition at all other temperatures tested.

TABLE 5. Effect of benzylpenicillin on cell division of *S. faecium* growing under different conditions

Temp (°C)	Benzylpenicillin MDC (μg/ml)	
	CDM	SB
30	8	0.5
32	4	0.2
37	4	0.2
42	0.5	0.016
45	0.04	0.004
47	0.01	0.004
49	0.01	ND[a]

[a] ND, Not determined.

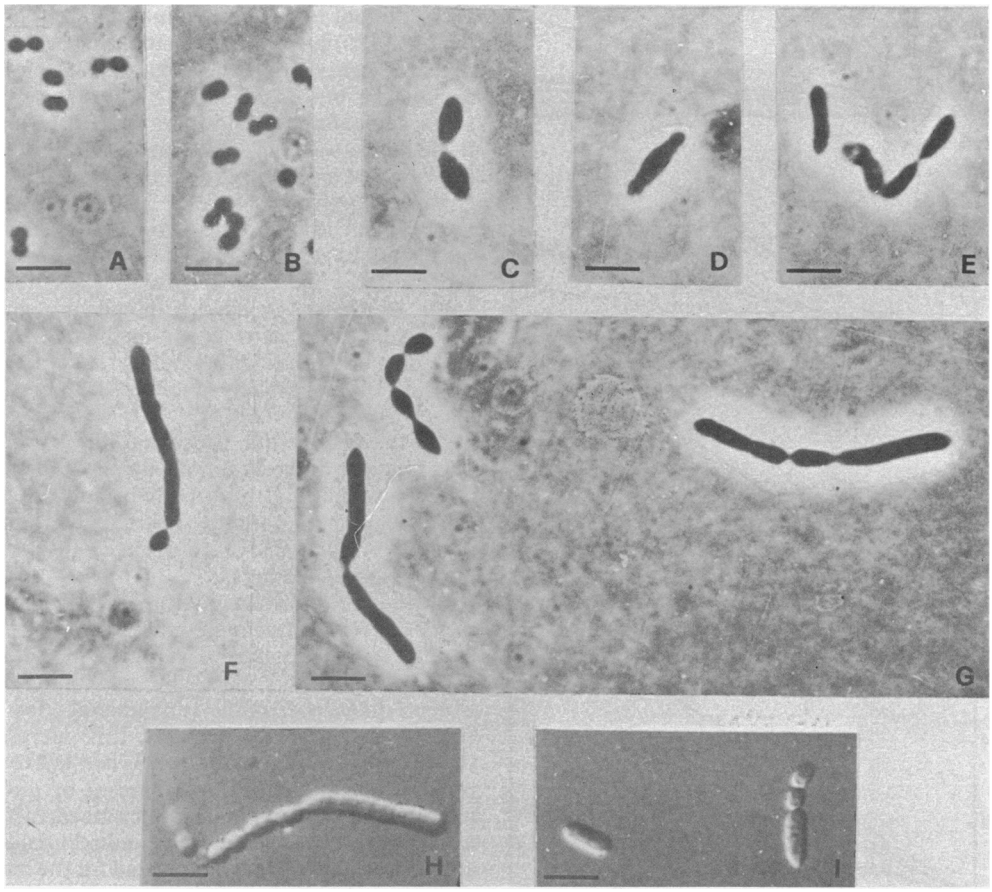

FIG. 2. *S. faecium* grown in the presence of benzylpenicillin under different conditions: (A) 32°C in CDM; (B) 45°C in CDM; (C, D, E) 32°C in CDM containing 4 μg of antibiotic per ml; (F, G) 45°C in CDM containing 0.04 μg of antibiotic per ml; (H) 37°C in SB containing 0.2 μg of antibiotic per ml; (I) 45°C in SB containing 0.004 μg of antibiotic per ml. A, B, C, D, E, F, and G are phase-contrast micrographs; H and I are interference phase-contrast micrographs. Bar, 0.7 μm.

DISCUSSION

The data presented here show that the susceptibility of *S. faecium* to penicillin (but not to other antibiotics of related and unrelated modes of action) is heavily influenced by both the composition of the medium and the growth temperature. Temperature and medium composition also influenced other physiological properties of the cells, such as generation time, autolysis, sensitivity of peptidoglycan synthesis to inhibition by penicillin, and the morphological effects of penicillin. However, no constant relation was found between the effects of growth conditions on any of these parameters and effects on penicillin susceptibility as expressed by the MIC. The pattern of labeling of PBPs was also found to be dramatically influenced by growth conditions.

We suggest that the status of PBPs may be heavily influenced by the physiological status of the cells and that as a consequence, the catalytic function of a given PBP may not always be connected to the same physiological activity of the cells. In other words, the effect which penicillin binding to PBPs has on cellular metabolism may depend on the specific physiological conditions of the bacteria, and the eventual inhibition of growth may occur by an indirect mechanism.

In *S. faecium*, the inhibition of peptidoglycan synthesis seems to be the most sensitive function associated with growth inhibition under some, but not all, the conditions tested. In cells incubated at 45°C in CDM, penicillin at the MIC inhibited peptidoglycan synthesis only slightly. In the same medium, peptidoglycan synthesis was much more sensitive to penicillin at 47°C than at 45°C, despite the fact that the cells had the same MIC at these two temperatures. This behavior may be explained by inhibition of cell

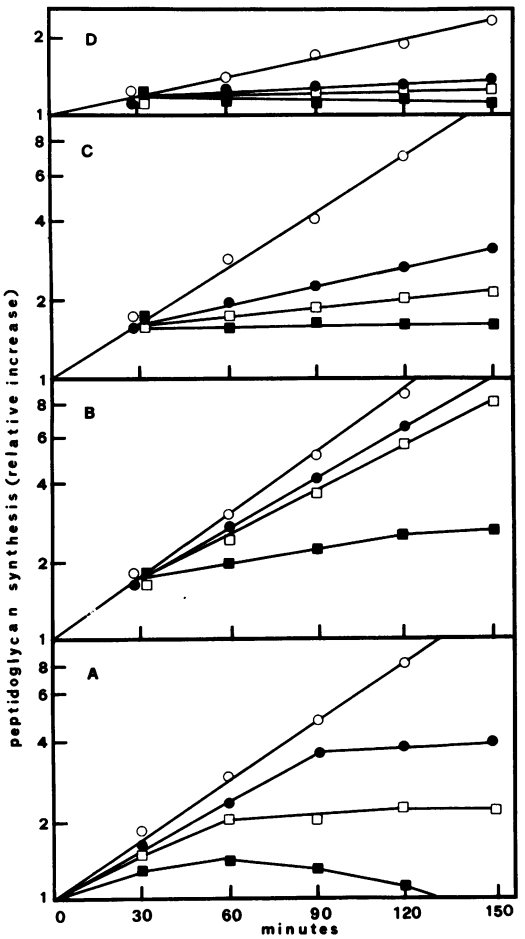

FIG. 3. Effect of penicillin on peptidoglycan synthesis. Cells exponentially growing in CDM at 32 (A), 45 (B), 47 (C), and 49°C (D) were treated with doses of penicillin corresponding to the MDC relative to each condition (●) and with doses twice (□) and four times (■) the MDC. Control cells (○) were also tested. At fixed intervals, incorporation of [^{3}H]lysine in trichloroacetic acid-precipitable material was determined.

envelope expansion as a cause of growth inhibition below 45°C. At 45°C, growth inhibition could result from specific inhibition of cell division. At 47°C, the growth rate is very slow, and cell surface expansion is probably no longer a primary requirement for cell growth since there are fewer polymers to be accommodated inside the envelope. The inhibitory effect under these conditions might therefore depend on mechanisms other than the inhibition of cell wall expansion and division.

Other findings presented in this study also support this proposal. Changes in penicillin susceptibility (growth inhibition) under different growth conditions did not appear related to the bacterial generation time but were associated with different PBP labeling patterns. Cells growing at 37°C in BHI broth and SB had quite different generation times (40 and 26 min, respectively) but were inhibited by exactly the same penicillin concentration; cells growing in these media with the same generation time of 38 min showed an over 200-fold difference in penicillin susceptibility (Table 1). The similarities in the PBP electrophoretic patterns in the three media at the temperatures allowing maximum growth rates indicate a specific physiological status of PBPs associated with cells growing at the fastest rate in a given medium. This is strongly supported by the finding that when cell division is specifically inhibited, rod-shaped bacteria are formed only by cells that are growing at the fastest rate. Since rod formation and cocci require different arrangements of the newly formed cell wall, this finding indicates that cell elongation is possible, upon septum inhibition, only when PBPs are engaged in specific physiological functions.

An indirect mechanism of penicillin action as proposed for *S. faecium* could explain the substantial species-to-species variation in the antibacterial effects of penicillin and could also explain differences in the antibacterial effects of structurally different β-lactams. It is interesting that a totally different approach has led to the proposal of an indirect mechanism of growth inhibition to explain the inhibitory mechanism of mecillinam (16). Models for the indirect mode of action have already been proposed for the mechanism of the irreversible effects (killing and lysis) of penicillin (18, 21).

ACKNOWLEDGMENTS

This work was supported by grants from the North Atlantic Treaty Organization (grant 1638), the Consiglio Nazionale delle Ricerche (contract 59/78.01874.65), Action Concertée Financée par l'État Belge (agreement 79/84-II), and Fonds National pour le Recherche Scientifique (J.C.).

We thank Carla Turcato for expert technical assistance and Donatella Rossi and Massimo Guida for secretarial assistance.

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