ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1987, p. 1150–1152
0066-4804/87/071150-03$02.00/0

Vol. 31, No. 7

Penicillin Tolerance in *Streptococcus faecium* ATCC 9790

I. SAID, H. FLETCHER, A. VOLPE,† AND L. DANEO-MOORE*

Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Received 3 October 1986/Accepted 29 April 1987

Tolerant strains of *Streptococcus faecium* had higher levels of muramidase 2 and lower levels of trypsin-activable muramidase 1 than did susceptible strains. Susceptible strains lysed faster than did tolerant strains in buffer and at some antibiotic concentrations. The addition of Triton X-100 produced equal lysis rates for susceptible and tolerant cultures.

We recently reported that *Streptococcus faecium* ATCC 9790 was tolerant (T) of the lethal action of penicillin G as well as of that of bacitracin, vancomycin, and D-cycloserine (8). Single-cell-colony isolates of ATCC 9790 exhibited an approximate 5% loss of tolerance. A lineage analysis over many transfers indicated that the change from tolerance to susceptibility was reversible and that susceptible (S) derivatives became tolerant at the same 5% frequency (8). The T and S cultures studied differed from each other in the extent of survival after exposure to penicillin G or to several other inhibitors of cell wall synthesis and also in the rate of penicillin-induced lysis (8). At concentrations that produced detectable lysis of S cultures, penicillin induced lysis of T cultures at a barely detectable rate. The availability of strains with a high rate of change between tolerant and susceptible states permitted an analysis of the physiological basis for tolerance. S and T derivatives recloned three times were used in this study.

Cultures were grown in Todd-Hewitt medium supplemented with 0.15% glucose, and antibiotics were added in the exponential phase of growth when the cultures contained about 10^8 cells per ml. All glassware was acid washed. For autolysis assay cells were harvested as described previously (14) by filtration on 0.65-μm-pore membrane filters (Millipore Corp., Bedford, Mass.), washed twice with about 5 ml (each time) of ice-cold distilled water, and suspended in 0.3 M potassium phosphate buffer (pH 6.8) (12). Autolysis rates (h^{-1}) were determined from the pseudo first-order reaction rate as described previously (14).

Table 1 shows the rates of lysis of exponential cultures exposed to various antibiotics. In the case of penicillin G or D-cycloserine, S cultures lysed faster than did T cultures at antibiotic concentrations below the MIC but not at or above the MIC. For bacitracin, S cultures lysed faster than did T cultures above the MIC. Finally, for vancomycin, rates of lysis were not very different for S and T cultures examined both above and below the MIC. The data shown in Table 1 indicate that in terms of cell lysis, T strains were only relatively tolerant, since high enough concentrations of each antibiotic resulted in lysis. For any one antibiotic, lysis was not necessarily initiated near the MIC, which was determined at a much lower inoculum and after 24 h of incubation. We have shown previously that the MICs of each of the above-mentioned antibiotics for S and T derivatives are essentially indistinguishable but that the MBCs are 16- to 100-fold higher for T derivatives than for S derivatives (8).

The rates of lysis of S and T derivatives were determined in 0.3 M potassium phosphate buffer. The T derivatives autolyzed more than two times more slowly than did the S derivatives (Table 2). The addition of trypsin to cells autolysing in 0.3 M potassium phosphate buffer substantially increased the rate of cell autolysis (Table 2), but the rates remained faster for the S derivatives than for the T derivatives. This result is in contrast to the marked stimulating effect of trypsin on the rates of lysis of several previously characterized autolysis-deficient derivatives of *S. faecium* ATCC 9790 (7, 16). Both the S and T derivatives had essentially the same rates of lysis after detergent treatment (Table 2).

Crude muramidase 1 (E1) and muramidase 2 (E2) were extracted from S and T cells by the methods described by Kawamura and Shockman (11, 15) and were assayed on *S. faecium* and *Micrococcus luteus* walls, respectively. In each instance the same dry weight of cells was suspended in the same volume for alkaline extraction. The results of seven

TABLE 1. Rates of lysis of S and T strains with various antibiotics[a]

Antibiotic and concn (μg/ml)	No. of determinations	Rate of lysis (h^{-1}) of:		S/T lysis rate ratio
		S strain	T strain	
Penicillin G				
5.0	14	0.64 ± 0.16	0.13 ± 0.07	4.9
10.0	8	0.89 ± 0.18	0.51 ± 0.19	1.8
20.0	10	0.83 ± 0.30	1.00 ± 0.63	0.8
D-Cycloserine				
1.0	4	0	0	
2.5	4	0.32 ± 0.03	0	∞
5.0	10	0.90 ± 0.37	1.02 ± 0.62	0.9
50.0	3	1.78 ± 1.25	3.03 ± 3.36	0.6
Bacitracin				
5.0	4	0	0	
10.0	4	0.26 ± 0.02	0.20 ± 0.04	1.3
20.0	4	0.73 ± 0.26	0.31 ± 0.06	2.4
25.0	4	1.19 ± 0.14	0.36 ± 0.09	3.3
Vancomycin				
0.25	3	0.23 ± 0.05	0.25 ± 0.02	0.9
2.5	10	0.29 ± 0.31	0.23 ± 0.23	1.3
25.0	4	0.39 ± 0.37	0.50 ± 0.11	0.8

[a] All determinations were done for pairs consisting of an S strain and a corresponding T strain. Rates of lysis were obtained from the exponential portion of the lytic response curve (14). The MICs determined in Todd-Hewitt medium containing glucose were as follows: penicillin G, 11 μg/ml; D-cycloserine, 39 μg/ml; bacitracin, 12 μg/ml; and vancomycin, 1.5 μg/ml (8). The MICs are ± 1 twofold dilution.

* Corresponding author.
† Present address: CENTOCOR, Inc., Malvern, PA 19355.

TABLE 2. Rates of lysis in potassium phosphate buffer

Buffer	Rate of lysis (h^{-1}) of:		S/T lysis rate ratio
	S strain	T strain	
Potassium phosphate buffer (0.3 M) alone	0.80	0.33	2.42
Buffer + trypsin (3 μg/ml)	3.19	0.96	3.32
Buffer + Triton X-100 (0.5%)	1.30	1.22	1.07
Buffer + Triton X-100 (0.8%)	2.77	2.60	1.07

independent extractions are given in Table 3. S and T cultures contained approximately equal levels of active E1; however, S cultures contained nearly twice as much trypsin-activable E1 as did T cultures. In contrast, T cultures contained twofold-higher levels of E2 than did S cultures.

In some instances, genetic defects in specific autolytic enzymes yield bacteria that are susceptible to growth inhibition but relatively resistant to the lytic and, to some extent, the bactericidal effects of some antibiotics (13, 18). In other instances, defects in specific autolytic enzymes result in slower rates of lysis and killing but not necessarily in tolerance (7, 16). The T derivatives reported here differed from the S derivatives in their muramidase content, but the differences were quantitative rather than qualitative. Moreover, although the T strains were deficient in trypsin-activable E1, they actually contained higher levels of E2 (Table 3). Thus, the reduced rates of autolysis are not readily explained.

Recently, Williamson and Tomasz reported that pneumococcal isolates that were apparently not deficient in autolytic activity but were tolerant of benzylpenicillin or of D-cycloserine plus β-chloroalanine were supersusceptible in terms of the initiation of lysis to either bacitracin or vancomycin (19). Two of their isolates, which had wild-type susceptibility to deoxycholate-induced lysis, were classified as Tol^+ Lyt^+. Susceptibility to deoxycholate-induced lysis was thought to distinguish the two isolates from Lyt^- organisms, which have defective autolytic activity (19). In autolysis-defective strains of *S. faecium*, susceptibility to deoxycholate or to Triton X-100 is essentially identical (16).

Both the T derivatives reported here and the Tol^+ mutants reported by Williamson and Tomasz (19) lysed after detergent treatment (Table 2). Unfortunately, the biochemical basis of this phenomenon is unclear. It is possible that the detergent treatment removes a lipidlike specific autolysin regulatory component from the cell membrane. The existence of such potentially regulatory components has been documented in both the pneumococcal and *S. faecium* autolytic systems (1–6, 9, 17). However, other explanations are possible, including destruction by the detergent of the integrity of the membrane proper. An intact membrane potential has been proposed to be necessary for the regulation of cell autolysis in *Bacillus subtilis* (10). The lysis of cultures of *S. faecium* deprived of glucose or exposed to fluoride is consistent with this second view (E. Ternove-Hinks, Ph.D. dissertation, Temple University, Philadelphia, Pa., 1977). A third possibility which cannot be discounted is that the detergent destroys the protoplast membrane and permits an uninhibited interaction between autolytic enzymes and their cell wall substrates. Whatever the mechanism, our data indicate that the detergent treatment removes the differences in the rates of lysis observed between our S and T strains.

TABLE 3. E1 and E2 levels in S and T cultures

Strain	U[a] of:			E2
	E1			
	Total[b]	Active	Activable[c]	
S	18.5 ± 5.2	5.8 ± 4.5	11.8 ± 6.7	6.8 ± 4.9
T	11.3 ± 4.6	5.7 ± 3.8	5.2 ± 4.1	13.3 ± 7.8

[a] A unit is defined as the amount which reduces the turbidity of the cell walls by 0.001 optical density units per min at 37°C. A total of 7 determinations were done for E2 and active E1, and 10 were done for total E1.

[b] Determined after the addition of trypsin (0.3 μg/ml).

[c] Determined as the difference between total and active E1 for each determination.

This work was supported by BRSG grant S07 RR05417 and by Public Health Service grant AI23394 from the National Institutes of Health.

LITERATURE CITED

1. **Carson, D., R. A. Pieringer, and L. Daneo-Moore.** 1979. Effect of growth rate on lipid and lipoteichoic acid composition in *Streptococcus faecium*. Biochim. Biophys. Acta **575**:225–233.
2. **Carson, D. D., and L. Daneo-Moore.** 1981. Cellular LTA content during growth and division in *Streptrococcus faecium* (ATCC 9790), p. 259–262. *In* G. D. Shockman and A. J. Wicken (ed.), Chemistry and biological activities of bacterial surface amphiphiles. Academic Press, Inc., New York.
3. **Carson, D. D., R. A. Pieringer, and L. Daneo-Moore.** 1981. Effect of cerulenin on cellular autolytic activity and lipid metabolism during inhibition of protein synthesis in *Streptococcus faecalis*. J. Bacteriol. **146**:590–604.
4. **Cleveland, R. F., L. Daneo-Moore, A. J. Wicken, and G. D. Shockman.** 1976. Effect of lipoteichoic acid and lipids on lysis of intact cells of *Streptococcus faecalis*. J. Bacteriol. **127**:1582–1584.
5. **Cleveland, R. F., J. V. Holtje, A. J. Wicken, A. Tomasz, L. Daneo-Moore, and G. D. Shockman.** 1975. Inhibition of bacterial wall lysins by lipoteichoic acids and related compounds. Biochem. Biophys. Res. Commun. **67**:1128–1135.
6. **Cleveland, R. F., A. J. Wicken, L. Daneo-Moore, and G. D. Shockman.** 1976. Inhibitors of wall autolysis in *Streptococcus faecalis* by lipoteichoic acid and lipids. J. Bacteriol. **126**: 192–197.
7. **Cornett, J. B., B. E. Redman, and G. D. Shockman.** 1978. Autolytic defective mutant of *Streptococcus faecalis*. J. Bacteriol. **133**:631–640.
8. **Daneo-Moore, L., A. Volpe, and I. Said.** 1985. Variation between penicillin-tolerant and penicillin-sensitive states in *S. faecium* ATCC 9790. FEMS Microbiol. **30**:319–323.
9. **Hackenbeck, R., S. Waks, and A. Tomasz.** 1978. Characterization of cell wall polymers secreted into the growth medium of lysis-defective pneumococci during treatment with penicillin and other inhibitors of cell wall synthesis. Antimicrob. Agents Chemother. **13**:302–311.
10. **Jolliffe, L. K., R. J. Doyle, and U. N. Streips.** 1981. Energized membrane and cellular autolysis in *Bacillus subtilis*. Cell **25**:753–763.
11. **Kawamura, T., and G. D. Shockman.** 1983. Purification and some properties of the endogenous, autolytic N-acetylmuramoyl-hydrolase of *Streptococcus faecium*, a bacterial glycoenzyme. J. Biol. Chem. **258**:9514–9521.
12. **Pooley, H. M., and G. D. Shockman.** 1969. Relationship between the latent form the active form of the autolytic enzyme of *Streptococcus faecalis*. J. Bacteriol. **100**:617–624.
13. **Sabath, L. D., N. Wheeler, M. Laverdiere, D. Blazevic, and B. J. Wilkinson.** 1977. A new type of penicillin resistance of *Staphylococcus aureus*. Lancet **i**:443–447.
14. **Sayare, M., L. Daneo-Moore, and G. D. Shockman.** 1972. Influence of macromolecular biosynthesis on cellular autolysis in *Streptococcus faecalis*. J. Bacteriol. **112**:337–344.
15. **Shockman, G. D., and T. Kawamura.** 1983. Evidence for the

presence of a second peptidoglycan hydrolase in *Streptococcus faecium*. FEMS Microbiol. Lett. **19**:65–69.
16. **Shungu, D. L., J. B. Cornett, and G. D. Shockman.** 1979. Morphological and physiological study of autolytic-defective *Streptococcus faecium* strains. J. Bacteriol. **138**:598–608.
17. **Shungu, D. L., J. B. Cornett, and G. D. Shockman.** 1980. Lipids and lipoteichoic acid of autolysis-defective *Streptococcus faecium* strains. J. Bacteriol. **142**:741–746.
18. **Tomasz, A., and M. Westphal.** 1971. Abnormal autolytic enzyme in a pneumococcus with altered teichoic acid composition. Proc. Natl. Acad. Sci. USA **68**:2627–2630.
19. **Williamson, R., and A. Tomasz.** 1980. Antibiotic-tolerant mutants of *Streptococcus pneumoniae* that are not deficient in autolytic activity. J. Bacteriol. **144**:105–113.