JOURNAL OF BACTERIOLOGY, Mar. 1993, p. 1717–1722
0021-9193/93/061717-06$02.00/0

Vol. 175, No. 6

Possible Role of a Choline-Containing Teichoic Acid in the Maintenance of Normal Cell Shape and Physiology in *Streptococcus oralis*

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Received 30 June 1992/Accepted 3 January 1993

***Streptococcus oralis* ATCC 35037 took up radioactively labeled choline from growth medium. Most of the choline (80 to 90%) was incorporated into the cell wall teichoic acid, and about 10% was localized in the plasma membrane. While cells grew in choline-free medium, they did so at slow rates and produced cell walls with greatly reduced amounts of phosphate and no detectable choline. Cells grown in choline-free medium had grossly abnormal shape and size. Both biochemical and morphological abnormalities were reversible by addition of choline to the medium.**

Teichoic acids, complex anionic wall polymers ubiquitous in gram-positive bacteria (30, 31), are known to have important ecological roles such as receptors for bacteriophage (1), as type- or group-specific antigens (7), and as reservoirs of surface-bound divalent cations (2, 31). Structurally similar so-called membrane teichoic acid polymers linked to glycolipids are also present in smaller quantities in the plasma membrane of all gram-positive bacteria. The precise physiological role(s) of these wall- and membrane-linked polymers in bacterial growth remains to be determined. Cell wall teichoic acids play a role in cellular morphogenesis in *Bacillus subtilis* and *Bacillus licheniformis* (3, 9, 10, 22), and genetic studies with teichoic acid mutants of *B. subtilis* imply that these polymers are essential for normal cell growth (5, 16, 19). Amino alcohols (e.g., choline), which are unique components of both the wall and membrane teichoic acids of *Streptococcus pneumoniae*, are essential nutrients for this bacterium (2, 27). Choline is also present in the wall teichoic acid of several viridans group streptococci and clostridia (20, 24, 26). *Streptococcus oralis* is frequently identified among viridans group streptococci that colonize human upper respiratory tracts (4, 20). While closely related to *S. pneumoniae*, this bacterium is classified as a distinct species on the basis of nucleic acid hybridization and cell wall composition data (20, 25, 26).

In preliminary studies with *S. oralis* ATCC 35037, it was noticed that omission of choline from a synthetic growth medium (28) caused striking abnormalities in the growth and morphology of these bacteria. In this communication, we describe an analysis of these phenomena.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. oralis* ATCC 35037 was cultivated in a chemically defined (CDen) medium, a synthetic medium (21) modified by replacing the Casamino Acid hydrolysate with a mixture of pure individual amino acids. The medium was modified as indicated in several experiments by omission of choline. Cultures of bacteria grown in choline-free medium were produced in the following manner. Bacteria grown in full (choline-containing) medium were diluted 100-fold into choline-free medium and grown at 37°C overnight, after which the culture was further diluted 10- to 20-fold into the same amino alcohol-free medium. The doubling time of cell mass was 1 h in the presence and about 2 to 2.5 h in the absence of choline. Cultures used for cell wall preparation were harvested at identical densities (optical density, 0.3 to 0.4) after at least 10 cell generations of growth in choline-free or choline-supplemented medium. Growth was monitored by measuring optical density at 620 nm with a spectrophotometer (Sequoia-Turner, Mountain View, Calif.). Stock cultures were frozen in CDen plus 10% glycerol and stored at −70°C.

Cell wall preparation. Cell walls were isolated from *S. oralis* by a published procedure (12, 13). Radioactively labeled cell walls were prepared from *S. oralis* grown in CDen with either L-[4,5-^{3}H,^{3}N]lysine (New England Nuclear Corp., Boston, Mass.), at 7.5 μCi/ml and a final concentration of 10 μg/ml, or [*methyl*-^{3}H]choline (Amersham Corp., Arlington Heights, Ill.), at 2 μCi/ml and a final concentration of 5 μg/ml, added to the medium. Cell walls were suspended in distilled water at a concentration of 10 mg/ml and stored at −20°C.

Enzymatic digestion of cell walls. Pneumococcal amidase was isolated from *Escherichia coli* CM 21 (11).

Cell walls labeled with [^{3}H]lysine (100 μg and 2×10^5 cpm, isolated from choline-grown cells; 100 μg and 3.5×10^4 cpm, isolated from cells deprived of choline) were suspended in 1 ml of 50 mM Tris-maleate buffer–0.1% Brij, pH 7. Pneumococcal amidase (0.2 μg/ml) was added, and the suspension was incubated at 37°C. Samples (150 μl) were removed at intervals, chilled on ice, and centrifuged in an Eppendorf microcentrifuge (Brinkmann Co., Teaneck, N.J.) at 12,000 × *g* for 10 min. Radioactivity released into the supernatant fluids was measured by placing 50-μl aliquots in 3 ml of Ready-safe scintillation fluid (Beckman Instruments, Inc., Fullerton, Calif.) and counting in a Mark II scintillation spectrometer. A similar method was used to digest cell walls with M1 muramidase (ICN, Irvine, Calif.), but the buffer used was adjusted to pH 5.6 (the optimal pH for the muramidase) and detergent was omitted.

Cell fractionation. Cells biosynthetically labeled in CDen with [^{3}H]choline were harvested and washed with saline. The cell pellet was suspended in 1/10 of a volume of H_2O and

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TABLE 1. Fractionation of [*methyl*-^{3}H]choline-labeled *S. oralis*[a]

Extraction	10^6 cpm in fraction (%)
Total cells	1.44 (100)
Cold TCA	0.018 (1.4)
$CHCl_3$-CH_3OH	<0.0004 (<1)
RNase-DNase solubilized	0.017 (1.2)
Trypsin solubilized	0.014 (1.0)
Pneumococcal amidase-M1 solubilized	1.28 (88.0)
Residual cpm (in plasma membrane ghost)	0.12 (8)

[a] *S. oralis* labeled with radioactive choline was fractionated as outlined in the text, and the radioactivity released into the supernatant fluids following each treatment was determined.

TABLE 2. Extraction of teichoic acid from radioactively labeled cell walls of *S. oralis*[a]

Treatment	% of total cell wall that was:	
	Choline labeled	Lysine labeled
TCA		
5%, 25°C, 24 h	10	1
5%, 60°C, 20 h	82	18
10%, 90°C, 20 min	98	17
Periodate (0.1 M, 37°C, 1 h)	82	5

[a] Purified radiolabeled cell walls were treated with TCA or periodate as described in the text. Following centrifugation in a microcentrifuge, the radioactivity present in the supernatant fluids was determined.

treated sequentially with the following: (i) trichloroacetic acid (TCA) (10% [wt/vol], 4°C, 20 min); (ii) an equal volume of chloroform plus methanol (2:1 [vol/vol]); (iii) RNase A and DNase I, 50 and 20 μg/ml, respectively, in 50 mM Tris-HCl (pH 7.5) plus 20 mM $MgSO_4$ (37°C, 1 h); (iv) trypsin at 100 μg/ml in 50 mM Tris-HCl (pH 8) plus 10 mM $CaCl_2$ (37°C, 2.5 h); and (v) pneumococcal amidase and M1 muramidase at 0.4 and 100 μg/ml, respectively, in 100 mM Tris-maleate (pH 6.9) (37°C, 20 min). The pellet was washed three times with the appropriate buffer between treatments. After the extractions, the sample was centrifuged at 20,000 × *g* for 30 min in a microcentrifuge to pellet the cell membranes. Radioactivity in each fraction was measured as described above.

NMR spectroscopy. Cell walls were solubilized with M1 muramidase prior to nuclear magnetic resonance (NMR) spectroscopy (13).

Extraction of teichoic acid from cell walls. Teichoic acids were extracted from the purified cell walls with hot TCA by the procedure described by Podvin et al. (24), which was modified by lowering the temperature to reduce peptidoglycan solubilization. Choline-labeled material was also released from cell walls by treatment with potassium periodate (23, 27).

Analytical procedures. The procedures for amino acid and amino sugar analyses of hydrolyzed cell walls and teichoic acids have previously been described (13). Phosphorus content of the cell walls was determined by the method of Chen et al. (8). Purified cell walls were acid hydrolyzed as described earlier (14, 15). The hydrolysates were then analyzed by thin-layer chromatography by using the solvent systems *n*-butanol–ethanol–acetic acid–water (8:2:1:3 [vol/vol]) and *n*-propanol–ammonia–water (6:3:1 [vol/vol]) as outlined by Hancock and Poxton (14).

Microscopy. Bacteria growing in choline-containing CDen or in choline-free CDen were observed by phase-contrast microscopy with a Carl Zeiss microscope at a magnification of ×1,250, and photographs were taken with a Nikon camera. Samples of the cultures were prepared for electron microscopy after fixation with glutaraldehyde (2%) (29).

RESULTS

Choline as a component of cell wall teichoic acid. Ninety percent of [^{3}H]choline incorporated by exponentially growing bacteria was found in the cell wall fraction, and 8 to 10% was associated with the cell membrane (Table 1). The latter

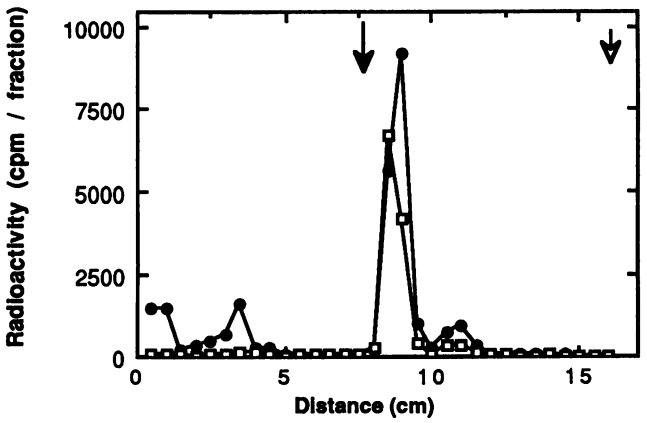

FIG. 1. Thin-layer chromatography of acid-hydrolyzed [*methyl*-^{3}H]choline-labeled *S. oralis* and *S. pneumoniae* cell walls. The chromatogram was developed, and the radioactivity of each 0.5-cm segment was assayed. The points of migration of the choline marker (closed arrow) and the solvent front (open arrow) are indicated. Hydrolyzed *S. oralis* walls are indicated by circles, and pneumococcal walls are indicated by squares.

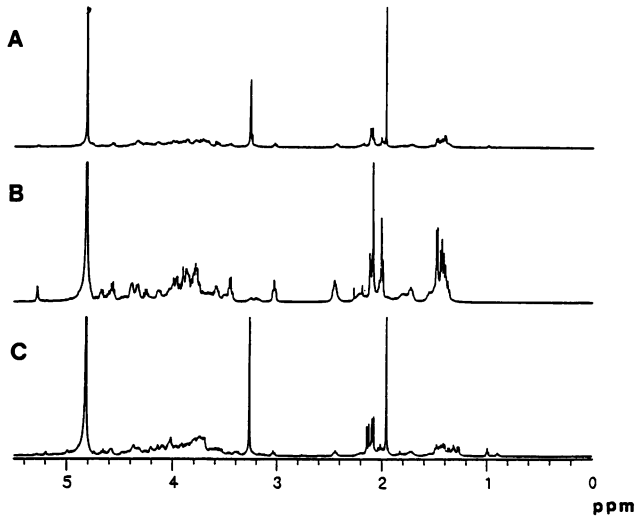

FIG. 2. Proton NMR spectra of M1 muramidase-digested *S. oralis* cell walls derived from cultures grown in the presence (A) or absence (B) of choline. The spectrum of a similar digest prepared from pneumococcal cell walls (C) is shown for comparison.

TABLE 3. Chemical analysis of *S. oralis* cell walls

Component	Concn (μmol/mg [dry wt]) of component[a] in:	
	Choline-grown cells	Cells grown without choline[b]
Alanine	0.79	0.80
Glutamic acid	0.55	0.57
Lysine	0.56	0.54
Glucosamine	1.03[c]	1.12[c]
Galactosamine	0.71[c]	0.67[c]
Choline	0.20[d]	ND[e]
Phosphorus	0.7	0.34

[a] The numbers shown are means derived from three independent cell wall preparations.
[b] The growth medium contained no amino alcohol.
[c] Approximate values uncorrected for decomposition during hydrolysis.
[d] Estimated from incorporation of [^{3}H]choline into cell walls.
[e] ND, not detectable by NMR spectroscopy.

material seems to be analogous to the Forssman antigen (membrane-associated lipoteichoic acid) of pneumococci (6, 17), since upon extraction of [^{3}H]choline-labeled protoplast membranes the bulk of the radioactivity was associated with the phenol phase, as is the case with pneumococcal Forssman antigen (data not shown). Virtually all of the incorporated label, following acid hydrolysis, was recovered as choline (Fig. 1), indicating the lack of metabolic conversion of the compound. Choline was specifically inserted into cell wall teichoic acid, since most of the label was extractable with hot TCA or periodate from isolated cell walls (Table 2). A smaller quantity of radioactive material was extracted by the same techniques when cell walls were labeled with [^{3}H]lysine instead of choline.

Teichoic acid composition of cell walls isolated from *S. oralis* grown in choline-free medium. In contrast to pneumococci, *S. oralis* grew in medium lacking choline. Cell walls derived from such cultures contained no choline detectable by NMR spectroscopy (Fig. 2). Further analyses of choline-free and choline-containing cell walls are shown in Table 3. The two types of cell walls showed similar amino acid and amino sugar contents. However, choline-free cell walls had approximately one-half of the normal level of phosphorus. Thin-layer chromatography (data not shown) demonstrated the presence of ribitol, galactose, and galactosamine in both wall preparations.

Cell walls derived from cells cultivated in choline-free medium were completely resistant to pneumococcal amidase (Fig. 3A)—an enzyme the hydrolytic activity of which was shown to have an absolute requirement for the presence of choline residues in the cell wall (15). The choline-free walls were, however, susceptible to another peptidoglycan hydrolase, M1 muramidase (Fig. 3B).

Abnormal growth of choline-deprived cells. Ability to grow without the choline supplement was a property of all of the cells, since *S. oralis* cultures plated on choline-containing and choline-free media produced the same number of CFU on agar. However, the growth rate of the bacteria was much slower when choline was omitted from the medium, as shown in Fig. 4. The slow growth was preceded by a long, variable lag phase. Adding choline to the culture medium

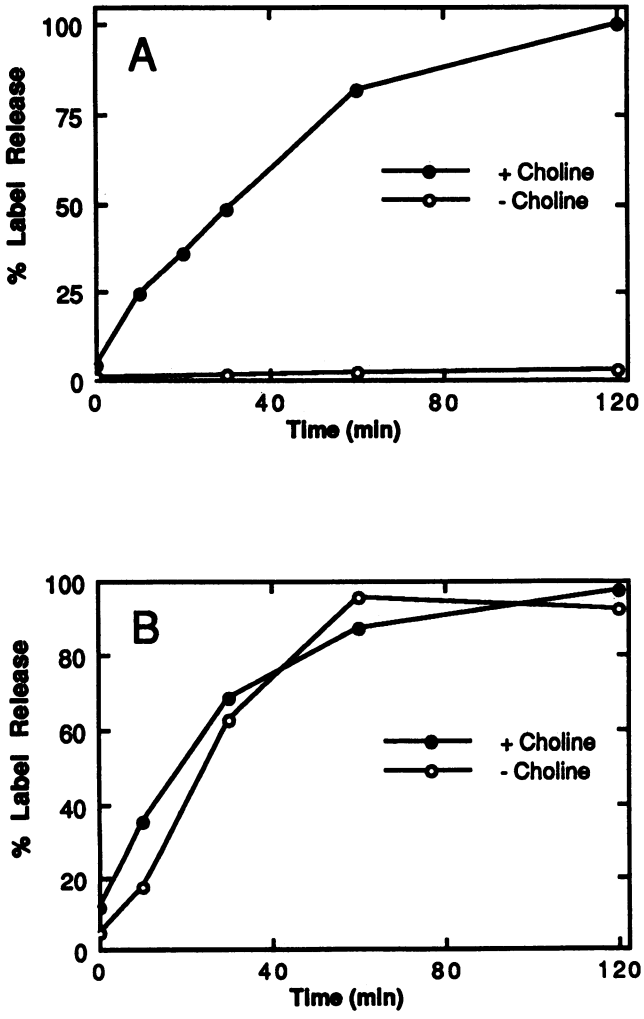

FIG. 3. Digestion of [^{3}H]lysine-labeled cell walls with pneumococcal amidase (A) and M1 muramidase (B). The assays were performed as described in the text, and the radioactivity released into the supernatant fluids was measured.

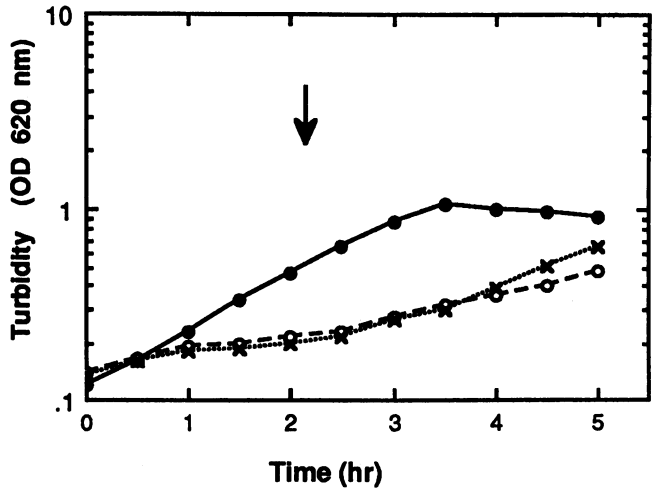

FIG. 4. Growth of *S. oralis* in CDen plus (●) and minus (○) choline. Optical densities (OD) of the cultures were measured at 30-min intervals. Choline was added to one culture grown without choline (×) at the time indicated by the arrow.

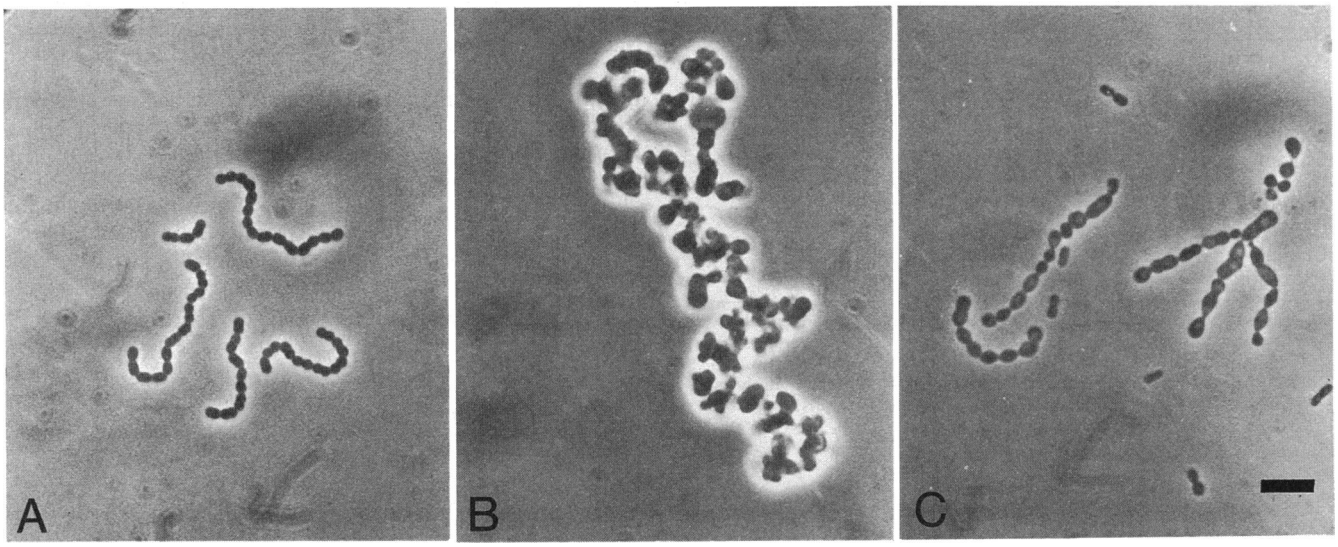

FIG. 5. Photomicrographs of *S. oralis* grown in the presence (A) or absence (B) of choline or in the absence of choline followed by addition of choline and incubation for another 9 h (C). Bar, 5 μm.

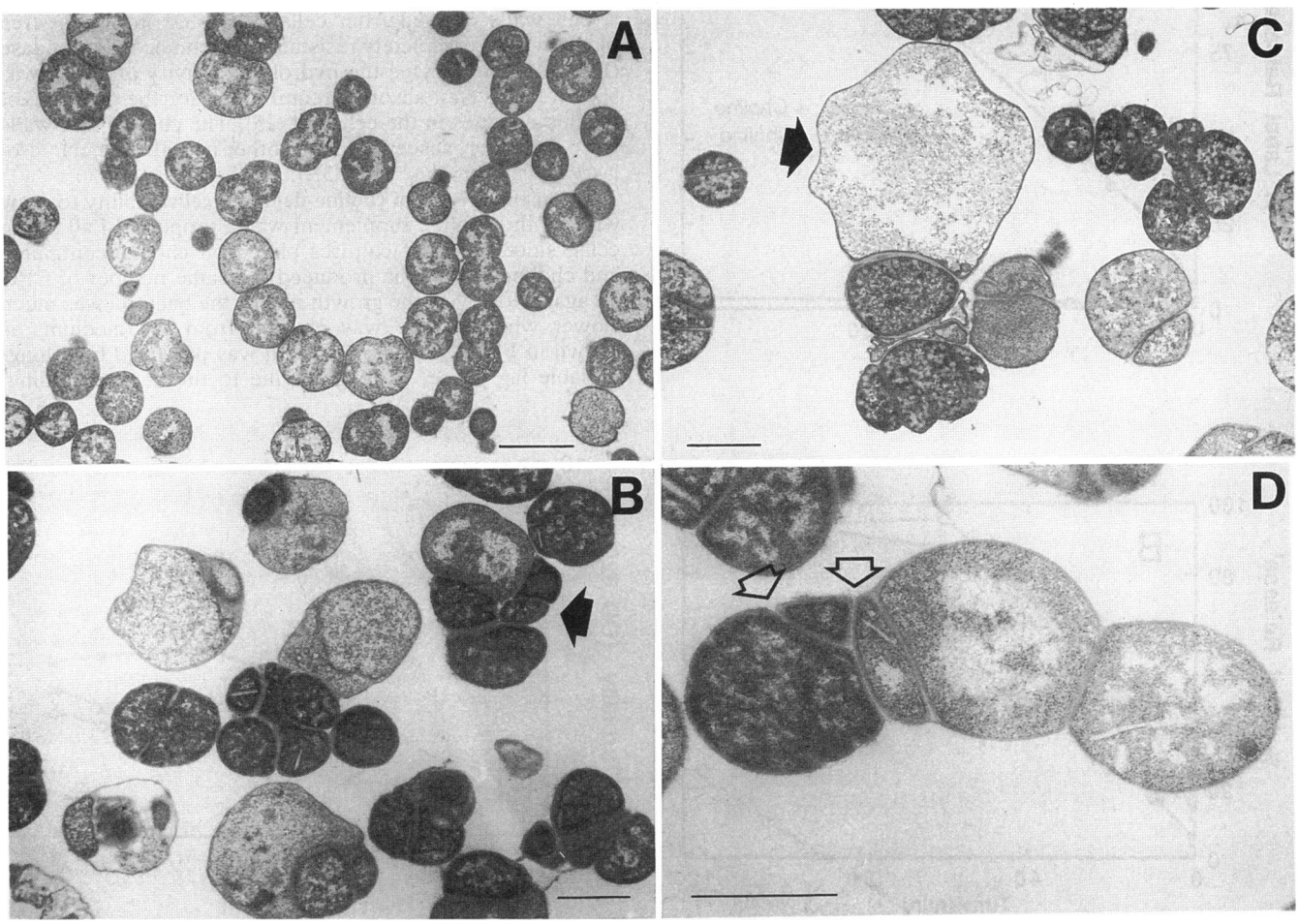

FIG. 6. Electron micrographs of thin sections of *S. oralis* grown with or without choline (bars, 1 μm). A, normal morphology of cells grown with choline. B, C, and D, cells grown for many generations without choline. Choline was added to the cells in panels B and D, which were incubated for an additional 5 h. The closed arrows in panels B and C illustrate abnormally small and large cells, respectively. The open arrows indicate septa inserted at oblique angles (D).

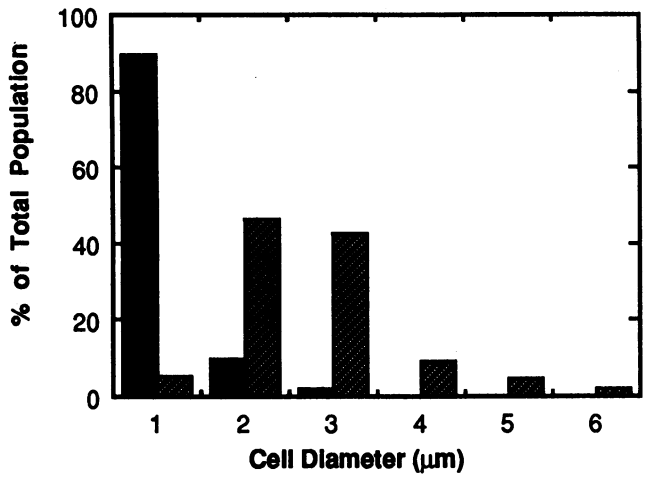

FIG. 7. Distribution of cell diameters in choline-grown (solid bars) and choline-free (cross-hatched bars) cultures. Cells growing exponentially with or without choline were observed under a phase-contrast microscope, the diameters of 1,000 cells were measured in each case, and the percentages of cells falling into the different categories were calculated.

resulted in resumption of normal growth within 3 h (Fig. 4) and renewed incorporation of [^{3}H]choline (data not shown).

Abnormal morphology in cells growing in the absence of choline. Bacteria grown with or without choline were observed by phase-contrast microscopy. Several morphological abnormalities were apparent in the choline-deprived cells. Unlike normal bacteria, which had a well-defined oval shape and were arranged in moderately long chains (Fig. 5A), choline-free bacteria (Fig. 5B) had irregular shapes and sizes and formed very long chains which folded back to form irregular clumps of cells. Adding choline to choline-deprived bacteria resulted in the gradual reappearance of normal morphology (Fig. 5C).

Electron micrographs show the anomalies in more detail. In contrast to the uniform size of normal cells (Fig. 6A), choline-free bacteria (Fig. 6B, C, and D) showed heterogeneous distribution from abnormally small to abnormally large sizes (Fig. 6B, C). Bacteria grown in choline-free medium showed a wide distribution of sizes, with the largest proportion of cells having diameters in the range of 2 to 3 μm (Fig. 7). In addition, choline-deprived bacteria seemed to have lost the ability to reproduce in normal shapes. Septa were frequently inserted at oblique angles (Fig. 6D, open arrows). Cells lacking these morphological aberrations appeared within 5 h after a shift back to choline-containing medium (Fig. 6B).

DISCUSSION

Like that of the taxonomically related pneumococci, the teichoic acid of *S. oralis* contains structure-bound choline, as well as ribitol, galactosamine, and phosphate (18, 25). In contrast to pneumococci, *S. oralis* did not show an auxotrophic requirement for choline, but cultures of *S. oralis* took up from the medium exogenous choline, the bulk of which was incorporated into the cell wall, while a smaller portion, approximately 8 to 10%, ended up in a membrane-bound form, similar to the membrane teichoic acid (Forssman antigen) of pneumococci (6).

Upon removal of choline from the medium, *S. oralis* grew after a prolonged and variable-length lag period and produced cell walls with greatly reduced phosphorus content and no detectable choline. The effect of choline deprivation on the membrane teichoic acid was not determined. Cells grown under these conditions also appeared to have lost control of cell size and shape. We suggest that these abnormalities are related to the abnormality of teichoic acid metabolism, supporting the proposition (5, 19) that anionic surface polymers perform vital functions in gram-positive bacteria.

Upon addition of choline to a choline-deprived culture, incorporation of choline resumed and normal growth rate and morphology were gradually restored. The reversibility of these phenomena may offer a new experimental system for the study of molecular steps in the control of the morphogenesis of these cocci.

ACKNOWLEDGMENT

These investigations were supported by a grant from the National Institutes of Health (RO1 AI16794).

REFERENCES

1. **Archibald, A. R.** 1980. Phage receptors in gram positive bacteria, p. 7–26. *In* L. L. Randall and L. Philipson (ed.), Virus receptors, part 1. Bacterial viruses (receptors and recognition, series B, vol. 7). Chapman & Hall, Ltd., London.
2. **Baddiley, J.** 1988. The functions of teichoic acids in walls and membranes of bacteria, p. 223–229. *In* H. Kleinhauf, H. von Dohren, and M. Jaenicke (ed.), The roots of modern biochemistry. Walter de Gruyter, Berlin.
3. **Boylan, R. J., N. H. Mendelson, D. Brooks, and F. E. Young.** 1972. Regulation of the bacterial cell wall: analysis of a mutant of *Bacillus subtilis* defective in biosynthesis of teichoic acid. J. Bacteriol. **110:**281–290.
4. **Bridge, P. D., and P. H. A. Sneath.** 1982. *Streptococcus gallinarum* sp. nov. and *Streptococcus oralis* sp. nov. Int. J. Syst. Bacteriol. **32:**410–415.
5. **Briehl, M., H. M. Pooley, and D. Karamata.** 1989. Mutants of *Bacillus subtilis* 168 thermosensitive for growth and wall teichoic acid and synthesis. J. Gen. Microbiol. **135:**1325–1334.
6. **Briles, E. B., and A. Tomasz.** 1973. Pneumococcal Forssman antigen: a choline containing lipoteichoic acid. J. Biol. Chem. **248:**6394–6397.
7. **Brundish, D. E., and J. Baddiley.** 1968. Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate. Biochem. J. **110:**573–582.
8. **Chen, P. S., T. Y. Toribara, and H. Warner.** 1956. Microdetermination of phosphorus. Anal. Chem. **28:**1756–1758.
9. **Ellwood, D. C., and D. W. Tempest.** 1969. Control of teichoic acid and teichuronic acid biosynthesis in chemostat cultures of *Bacillus subtilis* var. *niger*. Biochem. J. **111:**1–5.
10. **Forsberg, C. W., P. B. Wyrick, J. B. Ward, and H. J. Rogers.** 1973. Effect of phosphate limitation on the morphology and wall composition of *Bacillus licheniformis* and its phosphoglucomulase-deficient mutants. J. Bacteriol. **113:**969–984.
11. **Garcia, E., J.-L. Garcia, C. Ronda, P. Garcia, and R. Lopez.** 1985. Cloning and expression of the pneumococcal autolysin gene in *Escherichia coli*. Mol. Gen. Genet. **201:**225–230.
12. **Garcia-Bustos, J. F., B. T. Chait, and A. Tomasz.** 1987. Structure of the peptide network of pneumococcal peptidoglycan. J. Biol. Chem. **262:**15400–15405.
13. **Garcia-Bustos, J. F., and A. Tomasz.** 1987. Teichoic acid-containing muropeptides from *Streptococcus pneumoniae* as substrates for the pneumococcal autolysin. J. Bacteriol. **169:** 447–453.
14. **Hancock, I. C., and I. R. Poxton.** 1988. Accessory polymers of gram-positive bacteria, p. 179–190. *In* I. Hancock and I. Poxton (ed.), Bacterial cell surface techniques. John Wiley & Sons, Chichester, England.
15. **Holtje, J.-V., and A. Tomasz.** 1975. Specific recognition of

choline residues in the cell wall teichoic acid by the *N*-acetylmuramyl-L-alanine amidase of *Pneumococcus*. J. Biol. Chem. **250:**6072–6076.
16. **Honeyman, A. L., and G. C. Stewart.** 1988. Identification of the protein encoded by rod c, a cell division gene from *Bacillus subtilis*. Mol. Microbiol. **2:**735–741.
17. **Horne, D., and A. Tomasz.** 1985. Pneumococcal Forssman antigen: enrichment in mesosomal membrane and specific binding of the autolytic enzyme of *Streptococcus pneumoniae*. J. Bacteriol. **161:**18–24.
18. **Jenning, H. J., C. Lugowsky, and N. M. Young.** 1980. Structure of the complex polysaccharide C-substance from *Streptococcus pneumoniae* type 1. Biochemistry **19:**4712–4719.
19. **Karamata, D., H. M. Pooley, and M. Monod.** 1987. Expression of heterologous genes for wall teichoic acid in *Bacillus subtilis* 168. Mol. Gen. Genet. **207:**73–81.
20. **Kilpper-Bälz, P. Wenzig, and K. H. Schleifer.** 1985. Molecular relationships and classification of some viridans streptococci as *Streptococcus oralis* and emended description of *Streptococcus oralis* (Bridge and Sneath 1982). Int. J. Syst. Bacteriol. **35:**482–488.
21. **Lacks, S., and R. D. Hotchkiss.** 1980. A study of the genetic material determining an enzyme activity in *Pneumococcus*. Biochim. Biophys. Acta **39:**508–517.
22. **Mendelson, N. H.** 1982. Bacterial growth and division: genes, structures, forces, and clocks. Microbiol. Rev. **46:**341–375.
23. **Mosser, J. L., and A. Tomasz.** 1970. Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. J. Biol. Chem. **254:**287–298.
24. **Podvin, L., G. Reysset, J. Hubert, and M. Sebald.** 1988. Presence of choline in teichoic acid of *Clostridium acetobutylicum* N1-4 and choline inhibition of autolytic functions. J. Gen. Microbiol. **134:**1603–1609.
25. **Schmidhuber, S., R. Kilpper-Bälz, and H. K. Schleifer.** 1987. A taxonomic study of *Streptococcus mitis*, *S. oralis* and *S. sanguis*. Syst. Appl. Microbiol. **10:**74–77.
26. **Schmidhuber, S., W. Ludwig, and K. H. Schleifer.** 1988. Construction of a DNA probe for the specific identification of *Streptococcus oralis*. J. Clin. Microbiol. **26:**1042–1044.
27. **Tomasz, A.** 1967. Choline in the cell wall of a bacterium: novel type of polymer-linked choline in pneumococcus. Science **157:**694–697.
28. **Tomasz, A.** 1968. Biological consequences of the replacement of choline by ethanolamine in the cell wall of pneumococcus: chain formation, loss of transformability and loss of autolysis. Proc. Natl. Acad. Sci. USA **59:**86–93.
29. **Tomasz, A., J. D. Jamieson, and E. Ottolenghi.** 1964. The fine structure of *Diplococcus pneumoniae*. J. Cell Biol. **22:**453–480.
30. **Tomasz, A., M. Westphal, E. B. Briles, and P. Fletcher.** 1975. On the physiological functions of teichoic acids. J. Supramol. Struct. **3:**1–16.
31. **Ward, J. B.** 1981. Teichoic acid and teichuronic acids: biosynthesis, assembly, and location. Microbiol. Rev. **45:**211–243.