Pyrophosphate-Dependent Enzymes in Walled Bacteria Phylogenetically Related to the Wall-Less Bacteria of the Class *Mollicutes*[†]

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Some of the wall-less bacteria of the class *Mollicutes* (mycoplasmas) have pyrophosphate (PP_i)-dependent enzymic activities, including PP_i-dependent phosphofructokinase (PP_i-PFK), PP_i-dependent nucleoside kinase, and pyruvate, orthophosphate dikinase (PPDK) activities. In most other bacteria, adenosine 5'-triphosphate (ATP), not PP_i, is the cofactor of analogous enzymic reactions. Because PP_i-dependent enzymes are more common among mollicutes than other bacteria, we describe here an examination of the six walled bacteria that have been reported to be phylogenetically related to the mollicutes (*Clostridium innocuum*, *Clostridium ramosum*, *Erysipelothrix rhusiopathiae*, *Lactobacillus cateniformis*, *Lactobacillus vitulinus*, and *Streptococcus pleomorphus*) for PP_i-PFK, ATP-dependent PFK, phosphoenolpyruvate carboxytransphosphorylase, PPDK, and PP_i- and ATP-dependent acetate kinases. Two anaerobic mollicutes, *Anaeroplasma intermedium* and *Asteroleplasma anaerobium*, were also tested. *C. innocuum*, *E. rhusiopathiae*, *S. pleomorphus*, and *Anaeroplasma intermedium* had PP_i-PFK activities, whereas *C. ramosum*, the two lactobacilli, and *Asteroleplasma anaerobium* had only ATP-dependent PFK activities. *Asteroleplasma anaerobium* and all of the walled bacteria except *E. rhusiopathiae* had PPDK activities. All of the species except *Asteroleplasma anaerobium* and *E. rhusiopathiae* also had pyruvate kinase activities; the effects of allosteric activators were tested. Phosphoenolpyruvate carboxytransphosphorylase was detected by using two methods in *C. innocuum*, *C. ramosum*, and *S. pleomorphus*. All of the species tested had ATP-dependent acetate kinase activities, but none had detectable PP_i-dependent acetate kinase activity. The occurrence of one or more PP_i-dependent enzymes in the mollicutes and their walled relatives is a phenotypic indicator of the phylogenetic relatedness of these organisms. The distribution of these enzymes among members of this group substantiates the subgroups proposed by other workers who used 16S ribosomal ribonucleic acid analysis.

The wall-less eubacteria belonging to the genera *Mycoplasma*, *Ureaplasma*, *Spiroplasma*, *Acholeplasma*, *Anaeroplasma*, and *Asteroleplasma* are grouped together in the class *Mollicutes*. Phylogenetic analyses of 5S and 16S ribosomal ribonucleic acid (rRNA) sequences have revealed that the mollicutes cluster with the low-guanine-plus-cytosine content subdivision of gram-positive eubacteria that includes the bacillus-lactobacillus-streptococcus branch (31, 40, 41). Among the members of the low-guanine-plus-cytosine content gram-positive eubacteria, the mollicutes are most closely related to *Clostridium innocuum*, *Clostridium ramosum*, *Erysipelothrix rhusiopathiae*, *Lactobacillus cateniformis* (*Lactobacillus cateniforme*), *Lactobacillus vitulinus*, and *Streptococcus pleomorphus* (16, 31, 40-42; W. G. Weisburg, J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. Van Etten, J. Maniloff, and C. R. Woese, submitted for publication).

The mollicutes and their walled phylogenetic relatives have few phenotypic similarities. The only comparative study of the phenotypes of the mollicutes and their walled phylogenetic relatives was conducted by Pellegrin et al. (J. L. Pellegrin, J. Maugein, M. Clerc, B. Leng, and C. Bebear, Abstr. 7th Congr. Int. Organ. Mycoplasma., abstr.

no. P114, 1988). These authors found that *Clostridium innocuum*, *Clostridium ramosum*, and six species of mollicutes (belonging to four genera) were all resistant to high levels of rifampin. Generally, eubacteria are susceptible to rifampin, an inhibitor of ribonucleic acid polymerase; some mollicutes, however, are resistant (10).

Some members of the orders *Mycoplasmatales* and *Acholeplasmatales* have activities for (deoxy)ribonucleoside kinases that are dependent on pyrophosphate (PP_i) as a cofactor (18, 38), and *Acholeplasma* spp. have PP_i-dependent 6-phosphofructokinase (PP_i-PFK) activity (7, 23). Among the anaerobic mollicutes, both *Anaeroplasma intermedium* (18) and *Asteroleplasma anaerobium* (J. P. Petzel, M. C. McElwain, D. DeSantis, J. Manolukas, M. V. Williams, P. A. Hartman, M. J. Allison, and J. D. Pollack, Arch. Microbiol., in press) have activities for PP_i-dependent nucleoside kinases. *Anaeroplasma intermedium* also has PP_i-PFK activity, whereas *Asteroleplasma anaerobium* has activity for a third PP_i-dependent enzyme, pyruvate, orthophosphate dikinase (PPDK; Petzel et al., in press). PP_i-dependent nucleoside kinase has not been reported in any organism other than some mollicutes (18, 22, 38). Adenosine 5'-triphosphate (ATP) serves as the phosphate donor in the phosphofructokinase (PFK) reaction instead of PP_i in the great majority of bacteria. PPDK, which converts phosphoenolpyruvate (PEP) to pyruvate, has been reported in only a few procaryotes (43).

Because PP_i-dependent enzymes are generally rare among procaryotes but occur more frequently among the mollicutes, and because PP_i may be an evolutionary precursor of ATP (14, 43), we examined the walled phylogenetic relatives

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of the mollicutes and some members of the family *Anaeroplasmataceae* for the following four PP_i -dependent enzymes: PP_i -PFK, PPK, PEP carboxytransphosphorylase, and PP_i -dependent acetate kinase. We also assayed these bacteria for some analogous ATP-dependent enzyme activities.

MATERIALS AND METHODS

Strains. *Clostridium innocuum* ATCC 14501^T (T = type strain), *Lactobacillus cateniformis* ATCC 25536^T (= DSM 20559^T), *Lactobacillus vitulinus* ATCC 27783^T, and *Streptococcus pleomorphus* ATCC 29734^T (= DSM 20574^T) were obtained from the American Type Culture Collection, Rockville, Md. *Anaeroplasma intermedium* 5LA and 7LA^T (= ATCC 43166^T) (28, 30) and *Asteroleplasma anaerobium* 161^T (= ATCC 27880^T) (30) were obtained from I. M. Robinson, National Animal Disease Center, U.S. Department of Agriculture, Ames, Iowa. *Erysipelothrix rhusiopathiae* AVR-9 was obtained from R. L. Wood, National Animal Disease Center. *Clostridium innocuum* 7207 and *Clostridium ramosum* 8546 were received from the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg.

Cultivation and extract preparation. The mollicutes were harvested from 1.2 liters of S-2 broth ($\leq 0.5\%$ inocula; incubation for 4 to 6 days at 37°C) as described elsewhere (Petzel et al., in press). *Erysipelothrix rhusiopathiae* was grown (0.8% inoculum; incubation for 2 days at 37°C) in 1.2 liters of S-2 broth with the following modifications: the medium was supplemented with 1.7% (vol/vol) heat-inactivated horse serum and 10 mM MES [2-(N-morpholino)ethanesulfonic acid]; the lipids and Na_2CO_3 were deleted, and the culture was incubated without anaerobic precautions. All other species were grown in 800 ml of S-2 broth without the lipids (0.25% inocula; incubation for 20 to 22 h at 37°C).

Extracts of the mollicutes were prepared by osmotic lysis (Petzel et al., in press). Pellets of walled bacteria were suspended in the same lysis buffer and were passed through a French pressure cell two to four times. Lysates were clarified by centrifugation at $8,800 \times g_{max}$ and 4°C for 15 min and then at $230,000 \times g_{max}$ and 4°C for 1 h unless noted otherwise. Samples of the cell-free extracts were either frozen at -75°C or dialyzed against 100 volumes of lysis buffer (20 h, 4°C, one change) before being frozen. Some cell-free extracts were frozen under argon; no differences in activity were observed between these preparations and those with which no anaerobic precautions were taken. Protein concentrations were determined by using the BCA protein assay reagent (Pierce Chemical Co., Rockford, Ill.) and bovine serum albumin as the standard.

Enzyme assays. Biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo. All potentially labile substrates and cofactors were prepared in buffers and stored in the manner appropriate for maximum stability (2). ATP was determined by the method of Trautshold et al. (37).

ATP-dependent 6-phosphofructokinase (ATP-PFK) (EC 2.7.1.11) and PP_i -PFK (EC 2.7.1.90) were assayed in the forward direction by method B of O'Brien et al. (21). PP_i -PFK was also assayed in the reverse direction by method A (21). PPK (EC 2.7.9.1) was assayed by determining the appearance of ATP (8) [(NH₄)₆Mo₇O₂₄ was omitted] and by determining the appearance of pyruvate (9) (the concentration of MgCl₂ was decreased to 7.4 mM). PEP carboxytransphosphorylase (EC 4.1.1.38) was assayed by following the appearance of oxaloacetate, using a modifica-

tion of the method of Wood et al. (44). The mixture of KHCO₃, MgCl₂, and potassium phosphate buffer in a serum bottle was sparged with CO₂ for 30 min, the bottle was sealed with a septum, and samples were withdrawn with a syringe as needed. In addition, the concentration of CoCl₂ was reduced to 10 μ M, and dithiothreitol was substituted for β -mercaptoethanol. PEP carboxytransphosphorylase was also assayed by determining the appearance of PP_i ; the assay mixture used for the oxaloacetate assay was modified by deleting reduced nicotinamide adenine dinucleotide and malate dehydrogenase and increasing the concentration of CoCl₂ to 100 μ M. The reaction mixtures were incubated under CO₂ for 30 min at 37°C; mixtures without PEP served as controls. PP_i concentrations were determined with a commercial assay kit (Sigma) according to the directions of the manufacturer. Control values were subtracted from the values obtained by using complete assay mixtures. ATP-dependent acetate kinase (EC 2.7.2.1) reactions in the direction of acetyl phosphate formation were examined by using the method of Bowman et al. (3), with potassium acetate added to a concentration of 50 mM and sodium succinate (a positive effector in *Escherichia coli* [35]) added to a concentration of 10 mM. For each strain, a second assay mixture containing boiled cell-free extract was used as a blank for the spectrophotometric determination of acetyl hydroxamate by the method of Skarstedt and Silverstein (35). PP_i -dependent acetate kinase (EC 2.7.2.12) was determined similarly, except that the concentration of MgCl₂ was reduced to 0.75 mM to prevent precipitation with PP_i . Alternatively, to circumvent potential inhibition by PP_i of color formation in the hydroxylamine assay for acetyl phosphate in the PP_i -dependent acetate kinase reaction, the method of Reeves and Guthrie (25) was used. Sodium dithionite, which reportedly stimulates the reaction in *Desulfotomaculum ruminis* (15), was added to a concentration of 25 mM. Pyruvate kinase (EC 2.7.1.40) was assayed by using the method of Smart and Pritchard (36); PEP was added to a concentration of 4 mM. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed anaerobically by using the method described elsewhere (Petzel et al., in press).

RESULTS AND DISCUSSION

Weisburg and co-workers (Weisburg et al., submitted) used 16S rRNA analyses to determine that the walled bacteria which are phylogenetically related to the mollicutes can be divided into monophyletic subgroups. *Clostridium innocuum* clustered with *Streptococcus pleomorphus*, and *Clostridium ramosum* clustered with *Lactobacillus cateniformis* and *Lactobacillus vitulinus*. *Erysipelothrix rhusiopathiae* did not belong to either cluster, but Weisburg et al. (submitted) have reported that there were some data to suggest that *Erysipelothrix rhusiopathiae* is more closely related to the *Clostridium innocuum*-*Streptococcus pleomorphus* cluster than to the *Clostridium ramosum*-*lactobacillus* cluster.

The purpose of the present study was as follows: (i) to determine whether the walled bacteria that are related to the mollicutes possess PP_i -dependent enzymes as the mollicutes do, thereby providing a phenotypic indication of the phylogenetic relatedness of these organisms and (ii) to determine whether the distribution of PP_i -dependent enzymes among the six walled bacteria which we studied complements the phylogenetic subdivisions of this group proposed by Weisburg et al. (submitted).

Functional and structural patterns of various enzymes have been used as the bases for taxonomic and phylogenetic

classifications (4, 11, 39). Weitzman (39) reported that regulatory and size characteristics of citrate synthase and succinate thiokinase can be used to separate gram-positive from gram-negative bacteria and also aerobic gram-negative bacteria from facultative anaerobes. Jensen and co-workers (4, 11) have performed phylogenetic studies of various groups of bacteria and their pathways for aromatic amino acid biosynthesis by studying differences in isozymes, cofactor specificities, allosteric controls, and alternative biochemical steps; these authors determined that phylogenetic clusters of bacteria share distinctive biochemical features. However, these workers stressed that any conclusions about the phylogeny or evolution of organisms or their biochemical pathways must be made within the framework of phylogenetic relationships previously determined by other established methods, such as rRNA analysis (11).

The data reported in this paper represent enzyme activities determined in vitro (i.e., from cell extracts); thus, extrapolations to quantitative conclusions about conditions in vivo would be tenuous. When we could not detect an enzyme activity, when possible, enzyme from a commercial source or a known-positive organism was added to the putatively inactive cell-free extract to determine the efficacy of the assay system. Previous studies have demonstrated that, except for well-documented, isolated cases (see reference 1 for an example), most enzymes of carbohydrate metabolism (including PP_i-dependent enzymes) are not O₂ labile (6, 12, 26; Petzel et al., in press).

PFK. Among the walled bacteria, PP_i-PFK has previously been detected in a *Clostridium* sp. isolated from a cockroach gut (6), *Propionibacterium freundenreichii* and *Propionibacterium acidipropionici* ("Propionibacterium arabinosum") (21, 43, 45), some *Bacteroides* spp. (26), and *Deleya* spp. (33). Among the bacteria belonging to the class Mollicutes, PP_i-PFK has been detected in *Acholeplasma* spp., but the PFK activities among *Mycoplasma* spp. and *Spiroplasma* spp., when present, are active with ATP but not PP_i (7, 22, 23; M. H. V. Lake-Bullock, W. T. Blevins, S. K. McGlynn, and T. L. Rhoads, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, G-39, p. 155). In the anaerobic mollicutes, *Asteroleplasma anaerobium* 161^T has ATP-PFK activity, and *Anaeroplasma intermedium* 5LA has PP_i-PFK activity (Petzel et al., in press); PP_i-PFK activity has also been detected in *Anaeroplasma bactoclasticum* JR^T, the type species of the genus (Petzel, unpublished results).

In the present study, *Clostridium innocuum* and *Streptococcus pleomorphus* (one of the clusters within the group of six bacteria) had PP_i-PFK activities in both the forward and reverse directions (Table 1). However, *Clostridium ramosum*, *Lactobacillus cateniformis*, and *Lactobacillus vitulinus* (another cluster) had only ATP-PFK activity (Table 1). *Erysipelothrix rhusiopathiae* had PP_i-PFK activity (Table 1), in agreement with the phylogenetic evidence of Weisburg et al. (submitted) that suggests that *Erysipelothrix rhusiopathiae* is more closely allied with the *Clostridium innocuum* cluster than with the *Clostridium ramosum* cluster.

Parenthetically, PFK activities were sometimes detected in the two strains of *Clostridium innocuum*, in *Streptococcus pleomorphus*, and in the two strains of *Anaeroplasma intermedium* when ATP was substituted for PP_i; this was not observed with *Erysipelothrix rhusiopathiae*. This ATP-linked PFK activity was not detected in all extract preparations of these organisms. Indeed, in a previous study, we did not detect ATP-linked activity in *Anaeroplasma intermedium* 5LA (Petzel et al., in press). In the present study, the ATP-linked activities were always less than the PP_i-PFK

TABLE 1. PFK activities in walled bacteria phylogenetically related to members of the class Mollicutes and in anaerobic mollicutes

Species ^a	PFK activity (nmol/min per mg) ^b		
	PP _i -PFK		ATP-PFK
	Forward reaction	Reverse reaction	
<i>Clostridium innocuum</i> ATCC 14501 ^T	610	620	0.41 ^c
<i>Clostridium innocuum</i> 7207	260	340	14 ^c
<i>Streptococcus pleomorphus</i> ATCC 29734 ^T	650	740	14 ^c
<i>Clostridium ramosum</i> 8546	NA ^d	NA	68
<i>Lactobacillus cateniformis</i> ATCC 25536 ^T	NA	NA	200
<i>Lactobacillus vitulinus</i> ATCC 27783 ^T	NA	NA	53
<i>Erysipelothrix rhusiopathiae</i> AVR-9	920	960	NA
<i>Anaeroplasma intermedium</i> 5LA	380	190	39 ^c
<i>Anaeroplasma intermedium</i> 7LA ^T	120	80	4.2 ^c
<i>Asteroleplasma anaerobium</i> 161 ^T	NA	NA	37

^a Species are grouped according to the phylogenetic subdivisions determined by Weisburg et al. (submitted).

^b Data are the means of determinations from two batches of cells, except for *Anaeroplasma intermedium* 7LA^T and *Asteroleplasma anaerobium* 161^T (single batches of these strains were tested) (see below). See text for details of assays.

^c ATP-linked PFK activities in *Clostridium innocuum* ATCC 14501^T and 7207, *Streptococcus pleomorphus* ATCC 29734^T, and *Anaeroplasma intermedium* 7LA^T were detected in only one extract preparation; in *Anaeroplasma intermedium* 5LA, ATP-linked PFK activity was detected in four preparations. See text.

^d NA, No activity detected.

activities in these species (Table 1). Among the other microorganisms that have PP_i-PFK activities, *Clostridium* sp. (6), *Euglena gracilis* (19), *Propionibacterium* spp. (21, 45), and *Bacteroides* spp. (26) have been reported to also have ATP-PFK activities, but *Deleya* spp. has only PP_i-PFK activity (33). Reeves (24) initially reported that *Entamoeba histolytica* has both ATP- and PP_i-PFK activities. Subsequent investigations revealed that the seeming ATP-PFK activity resulted from the generation of PP_i from ATP by the glycogen-forming apparatus; strong centrifugal forces removed the ATP-linked activity from the extracts (24). Robertson and Glucina (26) reported that less ATP-PFK activity was detected in extracts of *Bacteroides* spp. subjected to a centrifugal force of 190,000 × *g* for 1 h than in extracts subjected to a centrifugal force of 27,000 × *g* for 20 min. The ATP-PFK activity of *Propionibacterium freundenreichii* was stable to a centrifugal force of 124,000 × *g* for 1 h (21). In the present study, we routinely used a centrifugal force of 230,000 × *g*_{max} for 1 h. We did not observe any marked reduction in ATP-linked activity in lysates of *Anaeroplasma intermedium* 5LA clarified by a centrifugal force of 330,000 × *g*_{max} for 120 min compared with lysates clarified at 27,000 × *g*_{max} for 20 min (data not shown).

To ensure that this ostensive ATP-PFK activity was not caused by contamination of assay components with PP_i (24), we performed the investigations described below. Using a PP_i assay kit (Sigma), we did not detect PP_i in either the ATP stock solutions or in cell-free extracts of *Anaeroplasma intermedium* 5LA. PP_i-PFK purified from *Propionibacterium freundenreichii* (Sigma) was not active with our ATP

TABLE 2. PPK activities in walled bacteria phylogenetically related to members of the class *Mollicutes* and in anaerobic mollicutes

Species ^a	PPDK activity (nmol/min per mg) ^b	
	Assay for ATP	Assay for pyruvate
<i>Clostridium innocuum</i> ATCC 14501 ^T	9.7	12
<i>Clostridium innocuum</i> 7207	13	12
<i>Streptococcus pleomorphus</i> ATCC 29734 ^T	30	30
<i>Clostridium ramosum</i> 8546	67	94
<i>Lactobacillus catenaformis</i> ATCC 25536 ^T	17	110
<i>Lactobacillus vitulinus</i> ATCC 27783 ^T	23	31
<i>Erysipelothrix rhusiopathiae</i> AVR-9	NA ^c	NA
<i>Anaeroplasma intermedium</i> 5LA	NA	NA
<i>Anaeroplasma intermedium</i> 7LA ^T	NA	NA
<i>Asteroleplasma anaerobium</i> 161 ^T	110	340

^a Species are grouped according to the phylogenetic subdivisions determined by Weisburg et al. (submitted).

^b Data are the means of determinations from two batches of cells (except for *Anaeroplasma intermedium* 7LA^T and *Asteroleplasma anaerobium* 161^T; single batches of these strains were tested). See text for details of assays.

^c NA, No activity detected.

solutions. To determine whether extracts of *Anaeroplasma intermedium* 5LA could generate PP_i from ATP, we assayed for the appearance of PP_i in ATP-PFK reaction mixtures containing all of the components except fructose 6-phosphate; no PP_i was detected. (Conversely, extracts of *Anaeroplasma intermedium* 5LA and *Clostridium innocuum* 7207 did not generate detectable amounts of ATP from PP_i.) Furthermore, the inclusion of 5 U of yeast inorganic pyrophosphorylase per ml of PFK assay mixture diminished PP_i-dependent activities, but not ATP-dependent activities, in *Anaeroplasma intermedium* 5LA and *Clostridium innocuum* 7207 (data not shown). These observations suggest the presence of bona fide ATP-PFK in *Clostridium innocuum*, *Streptococcus pleomorphus*, and *Anaeroplasma intermedium*; purification of the ATP-dependent activity from the PP_i-dependent activity would provide definite proof. The observations of the putative ATP-PFK aside, the occurrence of PP_i-PFK among some of the mollicutes and three of their six walled relatives is indicative of the phylogenetic relatedness of these organisms.

The occurrence of PP_i-PFK in *Acholeplasma* spp. (7, 23) and in *Anaeroplasma* spp. (Petzel et al., in press; this study) and the absence of PP_i-PFK in other mollicutes (including *Asteroleplasma anaerobium* 161^T) (7; Petzel et al., in press) correlate with the subgroups of mollicutes determined by Weisburg et al. (submitted) when 16S rRNA sequence analyses were used. These authors reported that *Acholeplasma laidlawii* and *Acholeplasma modicum* grouped with *Anaeroplasma* spp., to the exclusion of the other mollicutes.

PPDK and pyruvate kinase. PPDK converts PEP, adenosine 5'-monophosphate (AMP), and PP_i to pyruvate, ATP, and orthophosphate. This enzyme has previously been reported in *Propionibacterium freudenreichii*, *Propionibacterium acidipropionici*, *Clostridium symbiosum* ("Bacteroides symbiosus") some *Acetobacter* spp., the clostridium isolated from a cockroach gut, and three anoxygenic photosynthetic bacteria (6, 8, 9, 43, 45). Among the mollicutes, PPDK has been detected in *Asteroleplasma anaerobium* (Petzel et

TABLE 3. Effects of three potential allosteric activators on pyruvate kinase activities in walled phylogenetic relatives of members of the class *Mollicutes* and in anaerobic mollicutes

Species ^a	Pyruvate kinase activity (nmol/min per mg) with ^b :			
	No additives	0.5 mM Fru-1,6-P ₂	1.5 mM AMP	0.5 mM Glc-6-P
<i>Clostridium innocuum</i> ATCC 14501 ^T	7.0	10	49	130
<i>Clostridium innocuum</i> 7207	14	31	43	280
<i>Streptococcus pleomorphus</i> ATCC 29734 ^T	1.3	0.52	NA ^c	4.4
<i>Clostridium ramosum</i> 8546	20	19	200	540
<i>Lactobacillus catenaformis</i> ATCC 25536 ^T	NA	NA	260	NA
<i>Lactobacillus vitulinus</i> ATCC 27783 ^T	120	320	810	660
<i>Erysipelothrix rhusiopathiae</i> AVR-9	NA	NA	NA	NA
<i>Anaeroplasma intermedium</i> 5LA	170	160	170	180
<i>Anaeroplasma intermedium</i> 7LA ^T	81	85	90	80
<i>Asteroleplasma anaerobium</i> 161 ^T	NA	NA	NA	NA

^a Species are grouped according to the phylogenetic subdivisions determined by Weisburg et al. (submitted).

^b Data are the means of determinations from two batches of cells (except *Anaeroplasma intermedium* 7LA^T and *Asteroleplasma anaerobium* 161^T; single batches of these strains were tested). See text for details of assay.

^c NA, No activity detected.

al., in press) but not in any of the 10 other mollicutes examined (17).

In the present study, PPDK activity was detected in *Clostridium innocuum*, *Clostridium ramosum*, *Lactobacillus catenaformis*, *Lactobacillus vitulinus*, *Streptococcus pleomorphus*, and *Asteroleplasma anaerobium*, but not in *Erysipelothrix rhusiopathiae* or *Anaeroplasma intermedium* (Table 2). The activities were detected by assaying for both the conversion of PEP to pyruvate and the conversion of AMP to ATP. The fact that *Asteroleplasma anaerobium* is the only mollicute that has been reported to possess PPDK activity is phenotypic evidence that supports the unique phylogenetic status of this organism among the mollicutes (Weisburg et al., submitted). No conclusions about the specific relationship (if indeed any exists) between *Asteroleplasma anaerobium* and the walled relatives can be made on the basis of the present information concerning PPDK.

All of the walled relatives of the mollicutes except *Erysipelothrix rhusiopathiae* had pyruvate kinase activity, a second enzyme activity that interconverts PEP and pyruvate; the activity for *Streptococcus pleomorphus* was low and variable (Table 3). In previous studies, the pyruvate kinases of *Mycoplasma* spp., *Acholeplasma* spp., *Ureaplasma urealyticum*, and *Anaeroplasma intermedium* 5LA (5, 17; Petzel et al., in press) were detected in the absence of allosteric activators. In contrast, the pyruvate kinase activities in four of five walled bacteria determined in the present study to possess the enzyme were significantly increased by one or more activators (Table 3). Fructose 1,6-bisphosphate (Fru-1,6-P₂) had only moderate effects or no effect on the

pyruvate kinase activities of *Clostridium innocuum*, *Streptococcus pleomorphus*, and *Clostridium ramosum*; AMP and glucose 6-phosphate (Glc-6-P) were more effective.

Fru-1,6-P₂ activates pyruvate kinases in a wide variety of bacteria (32, 36, 46); fewer bacteria have pyruvate kinases activated by Glc-6-P but not Fru-1,6-P₂ (36, 46). Yamada and Carlsson (46) suggested that organisms that have both the Embden-Meyerhof-Parnas and pentose phosphate pathways for the degradation of glucose may use Fru-1,6-P₂ as an activator for pyruvate kinase because Glc-6-P is the branch point between the two pathways. However, organisms that lack the oxidative portion of the pentose phosphate pathway may use Glc-6-P as the primary activator for pyruvate kinase. We did not detect Glc-6-P dehydrogenase (the first enzyme in the oxidative portion of the pentose phosphate pathway) in cell-free extract preparations of any of the bacteria except *Streptococcus pleomorphus* (4.9 nmol/min per mg).

In the two lactobacilli, AMP was the best activator of pyruvate kinases; indeed, no activity was observed in extracts of *Lactobacillus cateniformis* in the absence of AMP (Table 3). Thus, the two lactobacilli differed from the third member of their phylogenetic subgroup, *Clostridium ramosum*. Weisburg et al. (submitted) have reported that although the lactobacilli cluster with *Clostridium ramosum*, they are more closely related to each other than to *Clostridium ramosum*.

The pyruvate kinase activity of *Anaeroplasma intermedium* was not significantly increased by any of the three effectors (Table 3). If the absence of allosteric activation of pyruvate kinase is confirmed for mollicutes in general, this would indicate that allosteric regulation of this enzyme arose in the walled relatives after the wall-less organism(s) diverged from the walled progenitor(s). The absence in *Asteroleplasma anaerobium* of pyruvate kinase activity, an enzyme activity detected in all other mollicute species examined, is further phenotypic evidence of the distinct phylogenetic status of this organism among the members of the class *Mollicutes*.

We could not detect either PPDK activity or pyruvate kinase activity in extracts of *Erysipelothrix rhusiopathiae* (Tables 2 and 3). The addition of Glc-6-P, Fru-1,6-P₂, or AMP had no effect. Robertson and McCullough (27) reported that *Erysipelothrix rhusiopathiae* degrades glucose via the Embden-Meyerhof-Parnas pathway, based on the distribution of ¹⁴C among products of [¹⁴C]glucose degradation. In the present study, the absence of detectable PPDK or pyruvate kinase activity in *Erysipelothrix rhusiopathiae* AVR-9 indicates either that our methods were not suitable for detecting these enzymes in this organism or that the avirulent vaccine strain used in the present study differed from strain S-192, which was used by Robertson and McCullough (27).

PEP carboxytransphosphorylase. PEP carboxytransphosphorylase occurs in *Propionibacterium freundenreichii*, *Propionibacterium acidipropionici*, some *Rhodopseudomonas* spp., *Brevibacterium ammoniagenes* ("Corynebacterium ammoniagenes"), and the clostridium isolated from a cockroach gut (6, 43-45). *Acholeplasma laidlawii* has been reported to possess PEP carboxytransphosphorylase activity (38; K. D. Beaman and J. D. Pollack, Yale J. Biol. Med. 57:897, 1984), but more recently, workers from the same laboratory have not been able to confirm this activity (17). In the present study, PEP carboxytransphosphorylase activities were detected by two methods in *Clostridium innocuum*, *Streptococcus pleomorphus*, and *Clostridium ramosum* (Ta-

TABLE 4. PEP carboxytransphosphorylase activities in walled bacteria phylogenetically related to members of the class *Mollicutes* and in anaerobic mollicutes

Species ^a	PEP carboxytransphosphorylase activity (nmol/min per mg) ^b	
	Assay for oxaloacetate	Assay for PP _i
<i>Clostridium innocuum</i> ATCC 14501 ^T	5.5	22
<i>Clostridium innocuum</i> 7207	22	81
<i>Streptococcus pleomorphus</i> ATCC 29734 ^T	27	25
<i>Clostridium ramosum</i> 8546	8.2	5.5
<i>Lactobacillus cateniformis</i> ATCC 25536 ^T	NA ^c	2.5
<i>Lactobacillus vitulinus</i> ATCC 27783 ^T	NA	0.71
<i>Erysipelothrix rhusiopathiae</i> AVR-9	NA	NA
<i>Anaeroplasma intermedium</i> 5LA	NA	7.4
<i>Anaeroplasma intermedium</i> 7LA ^T	NA	5.7
<i>Asteroleplasma anaerobium</i> 161 ^T	NA	NA

^a Species are grouped according to the phylogenetic subdivisions determined by Weisburg et al. (submitted).

^b Data are the means of determinations from two batches of cells (except for *Anaeroplasma intermedium* 7LA^T and *Asteroleplasma anaerobium* 161^T; single batches of these strains were assayed twice). See text for details of assays.

^c NA, No activity detected.

ble 4). Low levels of the enzyme were detected in *Lactobacillus cateniformis*, *Lactobacillus vitulinus*, and the two strains of *Anaeroplasma intermedium* by the method which detected PP_i, but not by the method which detected oxaloacetate (Table 4). No PEP carboxytransphosphorylase activity was detected in *Erysipelothrix rhusiopathiae* or *Asteroleplasma anaerobium* (Table 4). Thus, PEP carboxytransphosphorylase is an additional PP_i-dependent enzyme that is possessed by some of the walled relatives of the members of the class *Mollicutes*.

Acetate kinase. PP_i-dependent acetate kinase activities have been reported in *Entamoeba histolytica* (25) and in gram-negative sulfate-reducing anaerobes (15, 43). We did not detect PP_i-dependent acetate kinase activity in the direction of acetyl phosphate in any of the organisms which we examined; duplicate extracts of all organisms were tested by the modified method of Bowman et al. (3), and single extracts of *Clostridium innocuum* ATCC 14501^T, *Clostridium ramosum*, *Erysipelothrix rhusiopathiae*, *Lactobacillus cateniformis*, and *Streptococcus pleomorphus* were tested by using the method of Reeves and Guthrie (25). However, all of the species had activity for ATP-dependent acetate kinase in the direction of acetyl phosphate, as follows (means of determinations, in micromoles per minute per milligram, from two batches of cells, except as noted): *Clostridium innocuum* ATCC 14501^T, 132; *Clostridium innocuum* 7207, 355; *Streptococcus pleomorphus*, 228; *Clostridium ramosum*, 77.9; *Lactobacillus cateniformis*, 27.2; *Lactobacillus vitulinus*, 16.4; *Erysipelothrix rhusiopathiae*, 155; *Anaeroplasma intermedium* 5LA, 30.3; *Anaeroplasma intermedium* 7LA^T (single batch), 10.2; and *Asteroleplasma anaerobium* (single batch), 424. (We did not attempt to determine ATP-dependent acetate kinase activity in the direction of acetate and ATP synthesis because of the high levels of adenylate kinase activity in extracts of *Anaeroplasma intermedium* 5LA and *Asteroleplasma anaerobium* 161^T. Adenylate kinase, which converts 2 mol of adenosine

5'-diphosphate to 1 mol of ATP and 1 mol of AMP, interfered with the assay for ATP production in the ATP-dependent acetate kinase reaction.) Muhlrads et al. (20) did not detect ATP-dependent acetate kinase activity in *Anaeroplasma abactoclasticum* 6-1^T or *Anaeroplasma intermedium* 7LA^T. These researchers used the hydroxylamine method to detect acetyl phosphate production (20, 35), the same method used in the present study (3, 35). Kotzé (13) reported that the hydroxylamine method is subject to interference by substances in the sample and that it does not give reproducible results, but other researchers have used this method in extensive kinetic analyses of acetate kinases from *Veillonella alcalescens* (3) and *Escherichia coli* (35). Robinson and co-workers (28, 29) reported that acetate is produced from starch by *Anaeroplasma abactoclasticum* 6-1^T and *Anaeroplasma intermedium* 7LA^T and 5LA. Thus, the correlation between the production of acetate from starch and the production of acetyl phosphate from acetate and ATP suggests that these strains do possess acetate kinases.

Phylogenetic implications. The possession of a PP_i-dependent enzyme(s) by any two given species does not, by itself, necessarily denote a phylogenetic relationship between those species. For example, the bacteria examined in the present study and *Propionibacterium* spp. have several PP_i-dependent enzymes in common, but the propionibacteria belong to a different subdivision of gram-positive eubacteria, as determined by rRNA analysis (40). However, given that the bacteria examined in the present study are phylogenetically related to the mollicutes, the occurrence of one or more PP_i-dependent enzymes in each of these bacteria (enzymes that occur in other organisms infrequently) is phenotypic evidence that supports the phylogenetic relationship between the mollicutes and their walled relatives. Two unrelated clostridia, *Clostridium bifermentans* and *Clostridium butyricum*, did not have detectable PP_i-PFK or PPDK activities (data not shown).

Among mollicutes, the following four PP_i-dependent enzymes have been reported: PP_i-PFK, PPDK, PEP carboxytransphosphorylase, and PP_i-dependent nucleoside kinase. PP_i-dependent nucleoside kinase has been found in all three orders of the class Mollicutes (18, 22), whereas the other three enzymes have been reported only among members of the orders *Acholeplasmatales* and *Anaeroplasmatales* (7, 17; Petzel et al., in press; this study). The occurrence of multiple PP_i-dependent enzymes among the members of the orders *Acholeplasmatales* and *Anaeroplasmatales* and the walled phylogenetic relatives and the reported lack of any PP_i-dependent enzymes besides PP_i-dependent nucleoside kinase among the members of the order *Mycoplasmatales* support the rRNA sequence analyses that suggest that the achleoplasmas and anaeroplasmas represent the first branch(es) from the walled group (31, 40).

The findings reported in this paper are phenotypic indicators of the phylogenetic relatedness of the mollicutes to certain walled bacteria. The distributions of these enzymes substantiate the subdivision of this phylogenetic group as determined by 16S rRNA analyses. Characterization of these enzymes at the structural level (34) could help elucidate evolutionary lineages of the members of the class Mollicutes and their walled relatives.

LITERATURE CITED

1. Abbe, K., S. Takahashi, and T. Yamada. 1982. Involvement of oxygen-sensitive pyruvate formate-lyase in mixed-acid fermentation by *Streptococcus mutans* under strictly anaerobic conditions. *J. Bacteriol.* **152**:175-182.
2. Beutler, H.-O., and M. Supp. 1983. Coenzymes, metabolites, and other biochemical reagents, p. 328-393. In H. U. Bergmeyer, J. Bergmeyer, and M. Grassl (ed.), *Methods of enzymatic analysis*, 3rd ed., vol. 2. Samples, reagents, assessment of results. Verlag Chemie, Deerfield Beach, Fla.
3. Bowman, C. M., R. O. Valdez, and J. S. Nishimura. 1976. Acetate kinase from *Veillonella alcalescens*. *J. Biol. Chem.* **251**:3117-3121.
4. Byng, G. S., J. F. Kane, and R. A. Jensen. 1982. Diversity in the routing and regulation of complex biochemical pathways as indicators of microbial relatedness. *Crit. Rev. Microbiol.* **9**: 227-252.
5. Cocks, B. G., F. A. Brake, A. Mitchell, and L. R. Finch. 1985. Enzymes of intermediary carbohydrate metabolism in *Ureaplasma urealyticum* and *Mycoplasma mycoides* subsp. *mycoides*. *J. Gen. Microbiol.* **131**:2129-2135.
6. Cruden, D. L., W. E. Durbin, and A. J. Markovetz. 1983. Utilization of PP_i as an energy source by a *Clostridium* sp. *Appl. Environ. Microbiol.* **46**:1403-1408.
7. DeSantis, D., V. V. Tryon, and J. D. Pollack. 1989. Metabolism of mollicutes: the Embden-Meyerhof-Parnas pathway and the hexose monophosphate shunt. *J. Gen. Microbiol.* **135**:683-691.
8. Ernst, S. M., R. J. A. Budde, and R. Chollet. 1986. Partial purification and characterization of pyruvate, orthophosphate dikinase from *Rhodospirillum rubrum*. *J. Bacteriol.* **165**:483-488.
9. Evans, H. J., and H. G. Wood. 1971. Purification and properties of pyruvate phosphate dikinase from propionic acid bacteria. *Biochemistry* **10**:721-729.
10. Gadeau, A.-P., C. Mouches, and J. M. Bove. 1986. Probable insensitivity of mollicutes to rifampin and characterization of spiroplasmal DNA-dependent RNA polymerase. *J. Bacteriol.* **166**:824-828.
11. Jensen, R. A. 1985. Biochemical pathways in prokaryotes can be traced backward through evolutionary time. *Mol. Biol. Evol.* **2**:92-108.
12. Joyner, A. E., Jr., and R. L. Baldwin. 1966. Enzymatic studies of pure cultures of rumen microorganisms. *J. Bacteriol.* **92**: 1321-1330.
13. Kotzé, J. P. 1968. An enzymatic optical method for the determination of nanomole quantities of acetyl phosphate. *J. S. Afr. Chem. Inst.* **21**:105-112.
14. Kulaev, I. S., and V. M. Vagabov. 1983. Polyphosphate metabolism in micro-organisms. *Adv. Microb. Physiol.* **24**:83-171.
15. Liu, C.-L., and H. D. Peck, Jr. 1981. Comparative bioenergetics of sulfate reduction in *Desulfovibrio* and *Desulfotomaculum* spp. *J. Bacteriol.* **145**:966-973.
16. Ludwig, W., M. Weizenegger, R. Kilpper-Bälz, and K. H. Schleifer. 1988. Phylogenetic relationships of anaerobic streptococci. *Int. J. Syst. Bacteriol.* **38**:15-18.
17. Manolukas, J. T., M. F. Barile, D. K. F. Chandler, and J. D. Pollack. 1988. Presence of anaplerotic reactions and transamination, and the absence of the tricarboxylic acid cycle in mollicutes. *J. Gen. Microbiol.* **134**:791-800.
18. McElwain, M. C., D. K. F. Chandler, M. F. Barile, T. F. Young, V. V. Tryon, J. W. Davis, Jr., J. P. Petzel, C.-J. Chang, M. V. Williams, and J. D. Pollack. 1988. Purine and pyrimidine metabolism in Mollicutes species. *Int. J. Syst. Bacteriol.* **38**:417-423.
19. Miyatake, K., T. Enomoto, and S. Kitaoka. 1984. Detection and subcellular distribution of pyrophosphate: D-fructose 6-phosphate phosphotransferase (PFK) in *Euglena gracilis*. *Agric. Biol. Chem.* **48**:2857-2859.
20. Muhlrads, A., I. Peleg, J. A. Robertson, I. M. Robinson, and I. Kahane. 1981. Acetate kinase activity in mycoplasmas. *J. Bacteriol.* **147**:271-273.
21. O'Brien, W. E., S. Bowien, and H. G. Wood. 1975. Isolation and characterization of a pyrophosphate-dependent phosphofructokinase from *Propionibacterium shermanii*. *J. Biol. Chem.* **250**:8690-8695.
22. Pollack, J. D., M. C. McElwain, D. DeSantis, J. Manolukas, J. G. Tully, C.-J. Chang, R. F. Whitcomb, K. J. Hackett, and M. V. Williams. 1989. Metabolism of members of the *Spiroplasmataceae*. *Int. J. Syst. Bacteriol.* **39**:406-412.

23. Pollack, J. D., and M. V. Williams. 1986. PP_i-dependent phosphofructotransferase (phosphofructokinase) activity in the mollicutes (mycoplasma) *Acholeplasma laidlawii*. J. Bacteriol. **165**: 53–60.
24. Reeves, R. E. 1987. Metabolic energy supplied by PP_i, p. 255–259. In A. Torriani-Gorini, F. G. Rothman, S. Silver, A. Wright, and E. Yagil (ed.), Phosphate metabolism and cellular regulation in microorganisms. American Society for Microbiology, Washington, D.C.
25. Reeves, R. E., and J. D. Guthrie. 1975. Acetate kinase (pyrophosphate). A fourth pyrophosphate-dependent kinase from *Entamoeba histolytica*. Biochem. Biophys. Res. Commun. **66**: 1389–1395.
26. Robertson, A. M., and P. G. Glucina. 1982. Fructose 6-phosphate phosphorylation in *Bacteroides* species. J. Bacteriol. **150**:1056–1060.
27. Robertson, D. C., and W. G. McCullough. 1968. Glucose catabolism of *Erysipelothrix rhusiopathiae*. J. Bacteriol. **95**:2112–2116.
28. Robinson, I. M., and M. J. Allison. 1975. Transfer of *Acholeplasma bactoclasticum* Robinson and Hungate to the genus *Anaeroplasma* (*Anaeroplasma bactoclasticum* [Robinson and Hungate] comb.nov.): emended description of the species. Int. J. Syst. Bacteriol. **25**:182–186.
29. Robinson, I. M., M. J. Allison, and P. A. Hartman. 1975. *Anaeroplasma abactoclasticum* gen.nov., sp.nov.: an obligately anaerobic mycoplasma from the rumen. Int. J. Syst. Bacteriol. **25**:173–181.
30. Robinson, I. M., and E. A. Freundt. 1987. Proposal for an amended classification of anaerobic mollicutes. Int. J. Syst. Bacteriol. **37**:78–81.
31. Rogers, M. J., J. Simmons, R. T. Walker, W. G. Weisburg, C. R. Woese, R. S. Tanner, I. M. Robinson, D. A. Stahl, G. Olsen, R. H. Leach, and J. Maniloff. 1985. Construction of the mycoplasma evolutionary tree from 5S rRNA sequence data. Proc. Natl. Acad. Sci. USA **82**:1160–1164.
32. Sanwal, B. D. 1970. Allosteric controls of amphibolic pathways in bacteria. Bacteriol. Rev. **34**:20–39.
33. Sawyer, M. H., P. Baumann, and L. Baumann. 1977. Pathways of D-fructose and D-glucose catabolism in marine species of *Alcaligenes*, *Pseudomonas marina*, and *Alteromonas communis*. Arch. Microbiol. **112**:169–172.
34. Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam. 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. **51**:873–884.
35. Skarstedt, M. T., and E. Silverstein. 1976. *Escherichia coli* acetate kinase mechanism studied by net initial rate, equilibrium, and independent isotopic exchange kinetics. J. Biol. Chem. **251**:6775–6783.
36. Smart, J. B., and G. G. Pritchard. 1979. Regulation of pyruvate kinase from *Propionibacterium shermanii*. Arch. Microbiol. **122**:281–288.
37. Trautschold, I., W. Lamprecht, and G. Schweitzer. 1985. Adenosine 5'-triphosphate, UV-method with hexokinase and glucose-6-phosphate dehydrogenase, p. 346–357. In H. U. Bergmeyer, J. Bergmeyer, and M. Grassl (ed.), Methods of enzymatic analysis, 3rd ed., vol. 7. Metabolites 2. VCH Publishers, Deerfield Beach, Fla.
38. Tryon, V. V., and D. Pollack. 1984. Purine metabolism in *Acholeplasma laidlawii* B: novel PP_i-dependent nucleoside kinase activity. J. Bacteriol. **159**:265–270.
39. Weitzman, P. D. J. 1980. Citrate synthase and succinate thiokinase in classification and identification. Appl. Bacteriol. Symp. Ser. **8**:107–125.
40. Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. **51**: 221–271.
41. Woese, C. R., J. Maniloff, and L. B. Zablen. 1980. Phylogenetic analysis of the mycoplasmas. Proc. Natl. Acad. Sci. USA **77**:494–498.
42. Woese, C. R., E. Stackebrandt, and W. Ludwig. 1985. What are mycoplasmas: the relationship of tempo and mode in bacterial evolution. J. Mol. Evol. **21**:305–316.
43. Wood, H. G. 1985. Inorganic pyrophosphate and polyphosphates as sources of energy. Curr. Top. Cell. Regul. **26**:355–369.
44. Wood, H. G., J. J. Davis, and J. M. Willard. 1969. Phosphoenolpyruvate carboxytransphosphorylase from *Propionibacterium shermanii*. Methods Enzymol. **13**:297–309.
45. Wood, H. G., and N. H. Goss. 1985. Phosphorylation enzymes of the propionic acid bacteria and the roles of ATP, inorganic pyrophosphate, and polyphosphates. Proc. Natl. Acad. Sci. USA **82**:312–315.
46. Yamada, T., and J. Carlsson. 1975. Glucose-6-phosphate-dependent pyruvate kinase in *Streptococcus mutans*. J. Bacteriol. **124**:562–563.