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Sequence and organization of genes encoding enzymes involved in pyruvate metabolism in *Mycoplasma capricolum*

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Abstract

The region of the genome of *Mycoplasma capricolum* upstream of the portion encompassing the genes for Enzymes I and IIA^{glc} of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) was cloned and sequenced. Examination of the sequence revealed open reading frames corresponding to numerous genes involved with the oxidation of pyruvate. The deduced gene organization is *naox* (encoding NADH oxidase)-*lplA* (encoding lipoate-protein ligase)-*odpA* (encoding pyruvate dehydrogenase EIα)-*odpB* (encoding pyruvate dehydrogenase EIβ)-*odp2* (encoding pyruvate dehydrogenase EII)-*dldH* (encoding dihydrolipoamide dehydrogenase)-*pta* (encoding phosphotransacetylase)-*ack* (encoding acetate kinase)-*orfA* (an unknown open reading frame)-*kdtB-ptsI-crr*. Analysis of the DNA sequence suggests that the *naox* and *lplA* genes are part of a single operon, *odpA* and *odpB* constitute an additional operon, *odp2* and *dldH* a third operon, and *pta* and *ack* an additional transcription unit. Phylogenetic analyses of the protein products of the *odpA* and *odpB* genes indicate that they are most similar to the corresponding proteins from *Mycoplasma genitalium*, *Acholeplasma laidlawii*, and Gram-positive organisms. The product of the *odp2* gene contains a single lipoyl domain, as is the case with the corresponding proteins from *M. genitalium* and numerous other organisms. An evolutionary tree places the *M. capricolum odp2* gene product in close relationship to the corresponding proteins from *A. laidlawii* and *M. genitalium*. The *dldH* gene encodes an unusual form of dihydrolipoamide dehydrogenase that contains an aminoterminal extension corresponding to a lipoyl domain, a property shared by the corresponding proteins from *Alcaligenes eutrophus* and *Clostridium magnum*. Aside from that feature, the protein is related phylogenetically to the corresponding proteins from *A. laidlawii* and *M. genitalium*. The phosphotransacetylase from *M. capricolum* is related most closely to the corresponding protein from *M. genitalium* and is distinguished easily from the enzymes from *Escherichia coli* and *Haemophilus influenzae* by the absence of the characteristic amino-terminal extension. The acetate kinase from *M. capricolum* is related evolutionarily to the homologous enzyme from *M. genitalium*. Map position comparisons of genes encoding proteins involved with pyruvate metabolism show that, whereas all the genes are clustered in *M. capricolum*, they are scattered in *M. genitalium*.

Keywords: acetate kinase; dihydrolipoamide dehydrogenase; lipoate-protein ligase; NADH oxidase; PTS; phosphotransacetylase; pyruvate dehydrogenase

Mycoplasmas, the smallest free-living organisms, contain the least complex genomes (Razin, 1985, 1992). They appear to have evolved from Gram-positive bacteria by selective elimination of nonessential genes (Maniloff, 1983). Growth of these organisms requires complex media, reflecting the loss of many genes encoding anabolic enzymes, but retention of genes encoding catabolic pathways (Miles, 1992). *Mycoplasma capricolum* has a genome of 1,155 kb, sufficient to encode approximately 350 genes (Miyata et al., 1991).

It was shown previously (Cirillo, 1979) that *M. capricolum* can metabolize carbohydrates by the ubiquitous phosphoenolpyruvate:sugar phosphotransferase system (PTS) (for review, see Cirillo, 1979). This system promotes phosphotransfer from phosphoenolpyruvate to the heat-stable phosphocarrier protein HPr in a reaction proposed to be catalyzed by the homodimeric form of Enzyme I. Phosphorylated HPr then transfers a phosphoryl group to the sugar-specific acceptor proteins, referred to as Enzymes II. Each Enzyme II complex consists of one or two membrane embedded proteins or domains (IIC and IID), as well

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[1] Nucleotide sequence(s) reported in this paper has (have) been submitted to the GenBank™/EMBL Data Bank with accession number (U62057).

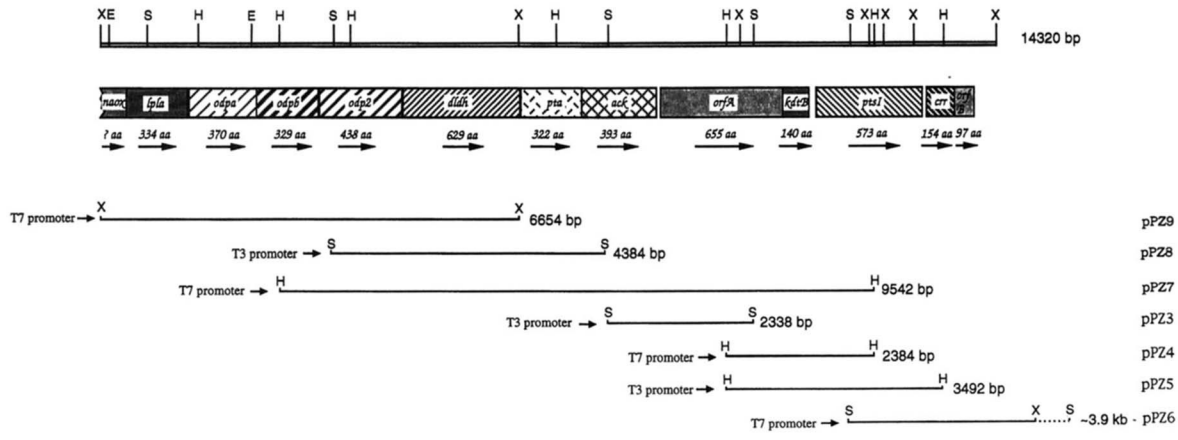


Fig. 1. Restriction and genetic maps of the region of the *M. capricolum* genome upstream of the ptsI-crr operon. The three clones that were isolated and sequenced are (**A**) pPZ7, the 9,542-bp clone derived from a partial *Hin*d III digest, (**B**) pPZ8, the 4,384-bp *Spe* I *clone*, (**C**) pPZ9, the 6,654-bp *Xba* I clone. The internal *Xba* I site is located at base 10209 (see Fig. 2). The internal *Eco*R I sites are located at bases 141 and 2396; The internal *Spe* I sites are located at bases 720, 3710, and 8094; the internal *Hin*d III sites are at bases 1551, 2834, 3966, 7266, and 9992. Horizontal arrows indicate direction of transcription of the indicated genes. --- aa, the number of amino acid residues of the deduced open reading frame. *naox*, the gene encoding NADH oxidase; *lplA*, the gene encoding lipoate-protein ligase; *odpA*, the gene encoding the EIα subunit of pyruvate dehydrogenase; *odpB*, the gene encoding the EIβ subunit of pyruvate dehydrogenase; *odp2*, the gene encoding the EII subunit of pyruvate dehydrogenase; *dldh*, the gene encoding dihydrolipoamide dehydrogenase; *pta*, the gene encoding phosphotransacetylase; *ack*, the gene encoding acetate kinase; *kdtB*, an open reading frame homologous to the *E. coli* KDTB protein product; *ptsI*, the gene encoding Enzyme I of the PTS; *crr*, the gene encoding enzyme IIA^{glc} of the PTS.

as two cytoplasmic proteins or domains (IIA and IIB) that pass the phosphoryl group from HPr to a specific sugar substrate during its translocation across the membrane (Postma et al., 1993).

In previous studies from this laboratory (Zhu et al., 1993, 1994), we reported the sequences of cloned *pts* genes from *M. capricolum*. It was shown that the *ptsH* gene, encoding HPr, constituted a unique monocistronic operon (the *ptsH* operon) in contrast to the typical occurrence in all *pts* operons sequenced to date of the *ptsH* gene immediately upstream of the *ptsI* gene, encoding Enzyme I. It was also established that *ptsI* is located in a dicistronic operon containing the gene encoding the glucose-specific Enzyme IIA (*crr*). In the present study, the region of the *M. capricolum* genome upstream of the *ptsI-crr* operon is characterized. It is shown that this region includes essentially all the genes necessary for the use of pyruvate.

Results

Cloning of the genes required for pyruvate metabolism

Previously we sequenced the region of the *M. capricolum* genome encoding the *ptsI-crr* operon (Zhu et al., 1994). Clones that were isolated in the course of that sequencing project were designated pPZ3, pPZ4, pPZ5, and pPZ6 (see Fig. 1). With the intention of investigating the organization of genes located upstream of the *ptsI-crr* operon, further clones were isolated. A partial digest with *Hin*d III provided DNA for the isolation of a 9,542-bp clone, designated pPZ7, which was screened using an oligonucleotide based on the pPZ4 sequence (from bases 3466 to 3422 of the sequence published previously) (Zhu et al., 1994) (see Materials and methods). Other DNA clones were isolated by a similar approach. pPZ8 was isolated from an *Spe* I digest and identified using an oligonucleotide sequence from pPZ7 corresponding to base numbers 8018–8062. The clone pPZ9 (from an *Xba* I digest) was also detected with a pPZ7 probe corresponding to bases 2882–2926 (see Fig. 1).

Sequence analysis of the genes required for pyruvate metabolism

Clones pPZ7, pPZ8, and pPZ9 were sequenced on both strands and then analyzed for open reading frames to reveal the gene organization illustrated in Figure 1. It was surprising to observe that a block of eight genes in this upstream region was in some way involved with the metabolism of pyruvate. The deduced gene organization, starting from the 5′-end of the sequence, is *naox* (encoding NADH oxidase), *lplA* (encoding lipoate-protein ligase), *odpA* (encoding the EIα subunit of pyruvate dehydrogenase), *odpB* (encoding the EIβ subunit of pyruvate dehydrogenase), *odp2* (encoding the EII subunit of pyruvate dehydrogenase), *dldH* (encoding dihydrolipoamide dehydrogenase), *pta* (encoding phosphotransacetylase), *ack* (encoding acetate kinase). The region downstream of *ack* (*orfA*, *kdtB*, *ptsI*, *crr*, and *orfB*) has been described previously (Zhu et al., 1994).

The combination of the three sequenced clones (pPZ7, pPZ8, and pPZ9) allowed us to deduce a total of 10,214 bp of DNA sequence (see Fig. 2). Bases 1–280 coded for an open reading frame that is homologous to the carboxyl-terminal region of the amino acid sequence of NADH oxidase from *Enterobacter faecalis* (Genbank accession no. P37061) and *M. genitalium* (MG275 from the TIGR database) (Fig. 3). Comparison (by BESTFIT analysis) of the *M. capricolum* and *E. faecalis* sequences indicated 64% similarity and 39% identity of the compared sequences over a length of 88 residues. When the *M. capricolum* sequence was compared with the *M. genitalium* se-

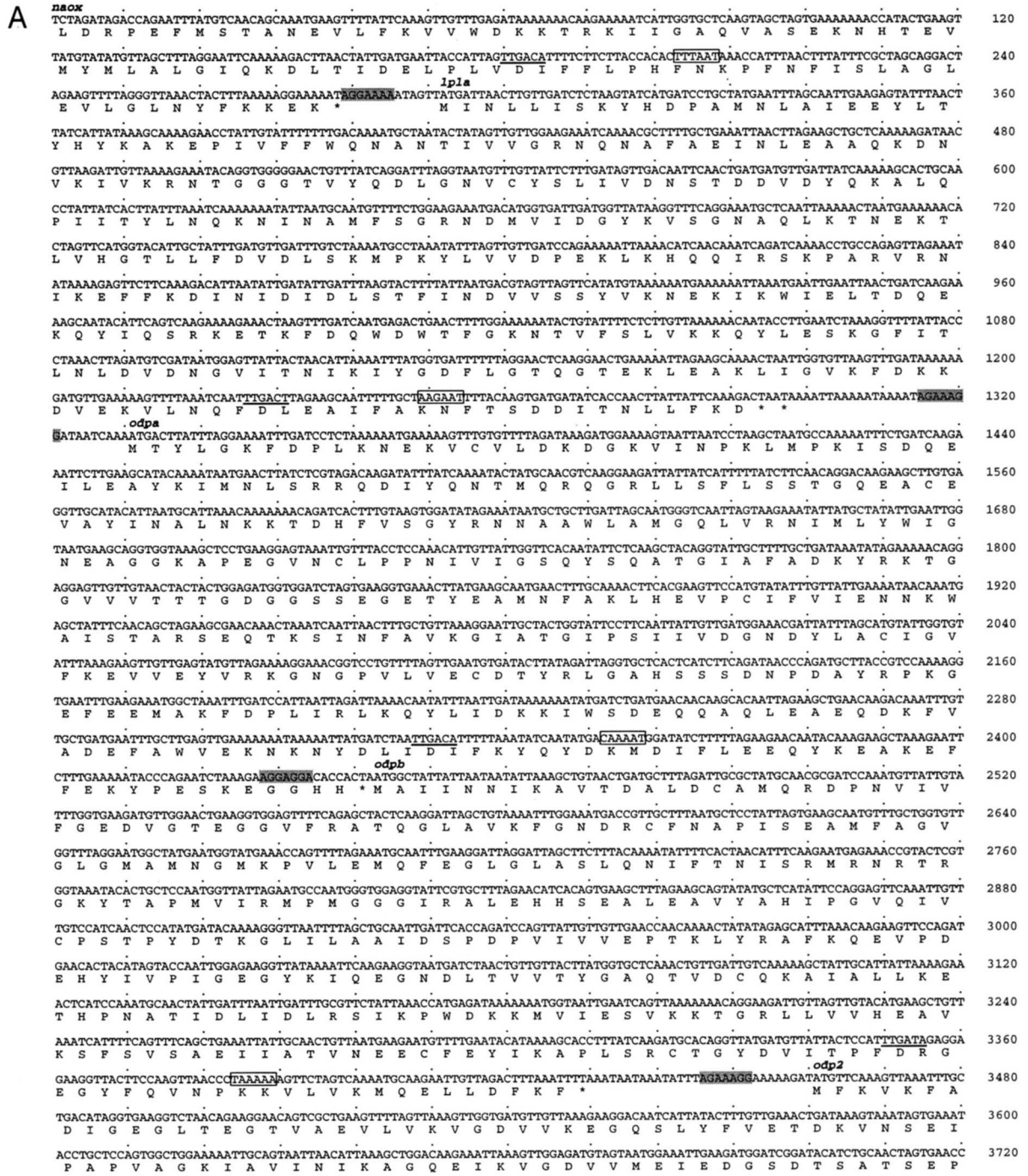


Fig. 2A. (*Figure continues on next two pages.*)

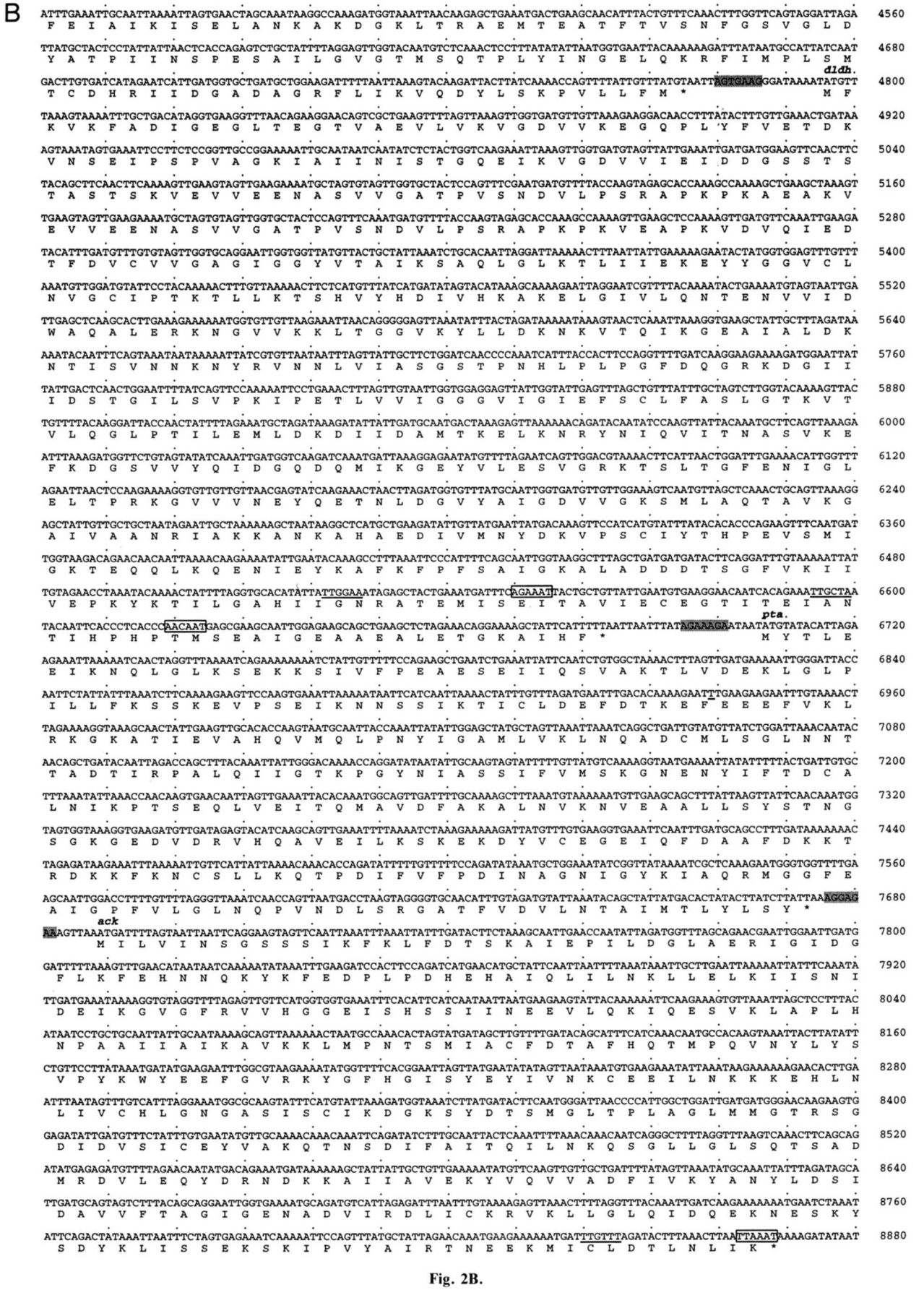


Fig. 2B.

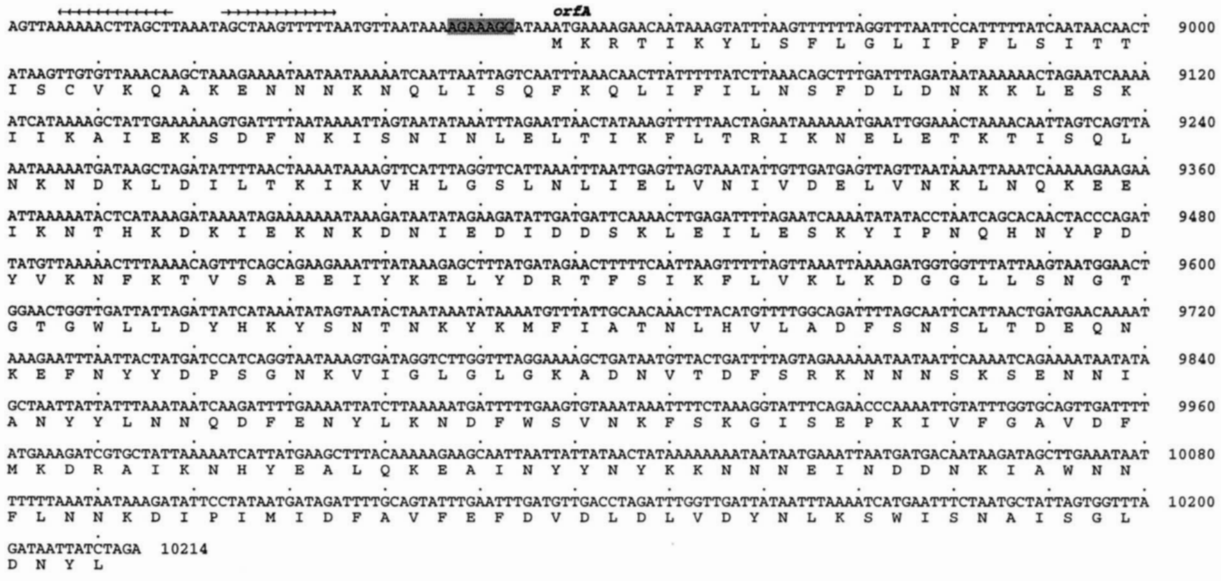


Fig. 2. Nucleotide sequence of the region of the *Mycoplasma* genome encoding genes involved with pyruvate metabolism.[1] A partial sequence of *naox* and the entire nucleotide sequences of *lplA*, *odpA*, *odpB*, *odp2*, *dldH*, *pta*, *ack*, and an ORF designated *orfA* (Zhu et al., 1994), as well as the deduced amino acid sequences, are shown. The putative promoter regions [i.e., 35 regions (underlined) and 10 regions (boxed)] are shown for the *lplA*, *odpA*, *odpB*, *odp2*, *pta*, and *orfA* genes. A sequence corresponding to a likely transcription termination site downstream of *ack* is marked with diverging arrows. Shine–Dalgarno ribosome binding sites for *lplA*, *odpA*, *odpB*, *odp2*, *dldH*, *pta*, *ack*, and *orfA* are highlighted with shaded boxes. Asterisks denote translation stop codons.

quence, it showed 77% similarity and 52% identity over a length of 92 residues.

After a gap of 11 bases, an open reading frame (from bases 292 to 1312) was identified. The end of this sequence was characterized by the presence of four in-frame stop codons (all TAA). Use of FASTA analysis of the deduced protein product led to its identification as the gene encoding lipoate–protein ligase. Alignment of the sequence with the lipoate–protein ligases from *M. genitalium* (MG270 from the TIGR database[2]) and *Escherichia coli* (Genbank accession no. P32099) is shown in Figure 4. BESTFIT analysis of the *Mycoplasma* sequence (334 amino acid residues) with the lipoate–protein ligase from *E. coli* (337 amino acid residues) showed 55% similarity and 32% identity over a length of 335 residues (data not shown). A similar analysis with the enzyme from *M. genitalium* (336 residues) showed 62% similarity and 36% identity over a length of 335 residues.

After a gap of 19 bases, an open reading frame (from bases 1331 to 2443) was observed. FASTA analysis showed the protein product to be the α-subunit of pyruvate dehydrogenase Enzyme I. Figure 5 shows an alignment of the sequence from *M. capricolum* with those from other sources recovered from the Genbank and TIGR databases. It is clear that the regions of total amino acid conservation (shown in reverse shading) are

[2] The *M. genitalium* sequence MG279 is listed in the TIGR database as unknown in function. The FASTA analysis reported here identifies the sequence as the gene encoding lipoate–protein ligase.

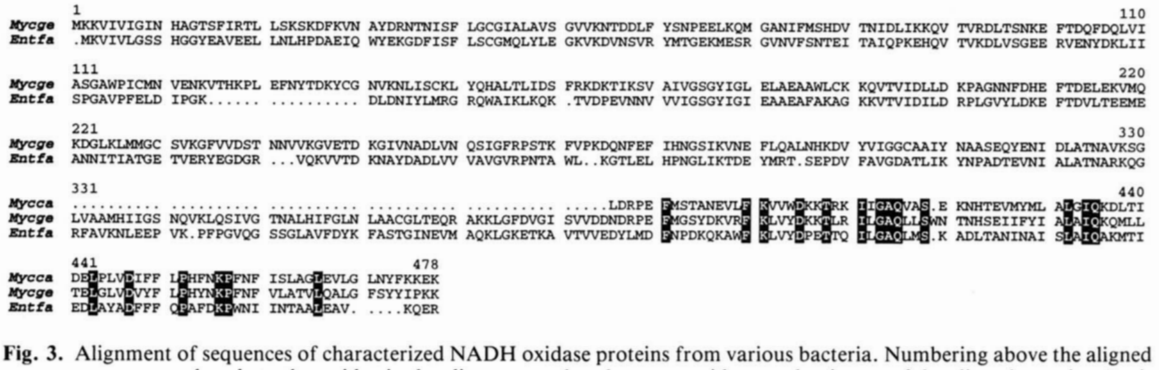


Fig. 3. Alignment of sequences of characterized NADH oxidase proteins from various bacteria. Numbering above the aligned sequences corresponds only to the residue in the alignment rather than to a residue number in any of the aligned proteins. Residues that are conserved in the listed NADH oxidase proteins are shown in reverse shading. Abbreviations used and references to published sequences are: *M. capricolum* (*Mcapr*) (this work); *M. genitalium* (*Mgeni*) (MG275 from the TIGR database); *E. faecalis* (*Entfa*) (Ross & Claiborne, 1992).

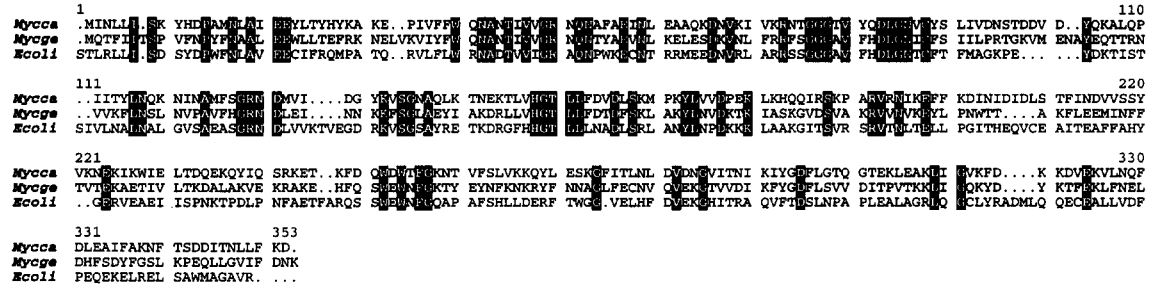


Fig. 4. Alignment of sequences of characterized lipoate-protein ligase proteins from various bacteria. Numbering above the aligned sequences corresponds only to the residue in the alignment rather than to a residue number in any of the aligned proteins. Residues that are conserved in the listed lipoate-protein ligase proteins are shown in reverse shading. Abbreviations used and references to published sequences are: *M. capricolum* (*Mcapr*) (this work); *M. genitalium* (*Mgeni*) (MG270 from the TIGR database); *E. coli* (*Ecoli*) (Morris et al., 1994).

Fig. 5 (*on next page*). Alignment of sequences of characterized Enzyme I-α proteins of the pyruvate and α-ketoacid dehydrogenases and acetoin catabolism complexes from various bacteria. Numbering above the aligned sequences corresponds only to the residue in the alignment rather than to a residue number in any of the aligned proteins. Residues that are conserved in all listed Enzyme I-α proteins are shown in reverse shading. Abbreviations used and references to published sequences are: *M. capricolum* (odpa-mycca) (this work); *M. genitalium* (odpa-mycge) (MG274 from the TIGR database); *A. laidlawii* (odpa-achla) (Wallbrandt et al., 1992); *B. subtilis* (odpa-bacsu) (Hemila et al., 1990) and (odba-bacsu) (Wang et al., 1993); *B. stearothermophilus* (odpa-bacst) (Borges et al., 1990); *A. thaliana* (odpa-arath) (Luethy et al., 1995); yeast (odpa-yeast) (Behal et al., 1989); human (odba-human) (McKean et al., 1992), (odpa-human) (Ho et al., 1989), and (odpt-human) (Dahl et al., 1987); pig (odpa-pig) (Sermon et al., 1990); mouse (odpa-mouse) (Fitzgerald et al., 1992) and (odpt-mouse) (Fitzgerald et al., 1992); rat (odba-rat) (Zhang et al., 1987), (odpt-rat) (Cullingford et al., 1993), and (odpa-rat) (Cullingford et al., 1994); *Ascarus suum* (odpt-ascu) (Johnson et al., 1992) and (odpa-ascu) (Johnson et al., 1992); *C. magnum* (acoa-cloma) (Kruger et al., 1994); *A. eutrophus* (acoa-alceu) (Priefert et al., 1991); *Pelobacter carbinolicus* (acoa-pelca) (Oppermann & Steinbuchel, 1994); *K. pneumoniae* (acoa-klepn) (Deng et al., 1994); bovine (odba-bovin) (Hu et al., 1988); *Pseudomonas putida* (odba-psepu) (Burns et al., 1988). odpa and odpt designations correspond to pyruvate dehydrogenase, whereas odba designations correspond to α-keto acid dehydrogenase complex gene products, respectively, and the acoa designation corresponds to acetoin catabolism complexes.

also shared by the sequence from *M. capricolum*. The signature sequence (shown as residues 250–280) G(D/E) (G/A) (X26)NN, characteristic of thiamine diphosphate-dependent enzymes, is also found in the *M. capricolum* sequence. A phylogenetic tree (Fig. 6) of the sequences of the α-subunit family shows that the *M. capricolum* protein is related closely to the corresponding proteins from *M. genitalium*, *Acholeplasma laidlawii*, and those from Gram-positive organisms (*Bacillus subtilis* and *B. stearothermophilus*).

The last base of the termination codon (TAA) of the sequence encoding the α-subunit of pyruvate dehydrogenase Enzyme I is also the first base of an open reading frame (from bases 2443 to 3432) that codes for the β-subunit of pyruvate dehydrogenase Enzyme I. Figure 7 shows an alignment of the sequence from *M. capricolum* with those from other sources recovered from the Genbank and TIGR databases. The alignment shows that residues that are totally conserved (shown as reverse shading) are also identical in the sequence from *M. capricolum*. It is noteworthy that all the bacterial sequences are from 40 to 70 residues shorter at the amino-terminal end than those sequences from eukaryotes. A phylogenetic tree (Fig. 8) shows a similar pattern to that observed for ODPA; the protein from *M. capricolum* is most closely related to those from *M. genitalium* (MG273, TIGR, database) and *A. laidlawii*.

After a gap of 28 bases, a new open reading frame (from bases 3461 to 4877), corresponding to the Enzyme II subunit of pyruvate dehydrogenase, was identified. An alignment of the *M. capricolum* sequence with other Enzyme II sequences (Fig. 9) shows clearly that the conserved residues (reverse shading) are found in the *M. capricolum* sequence. Boxes shaded in grey cor-

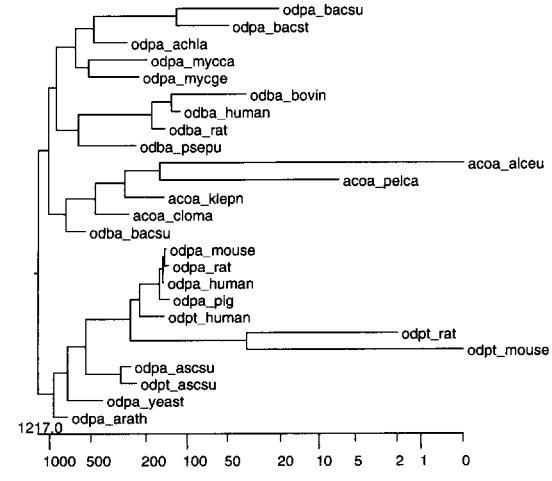
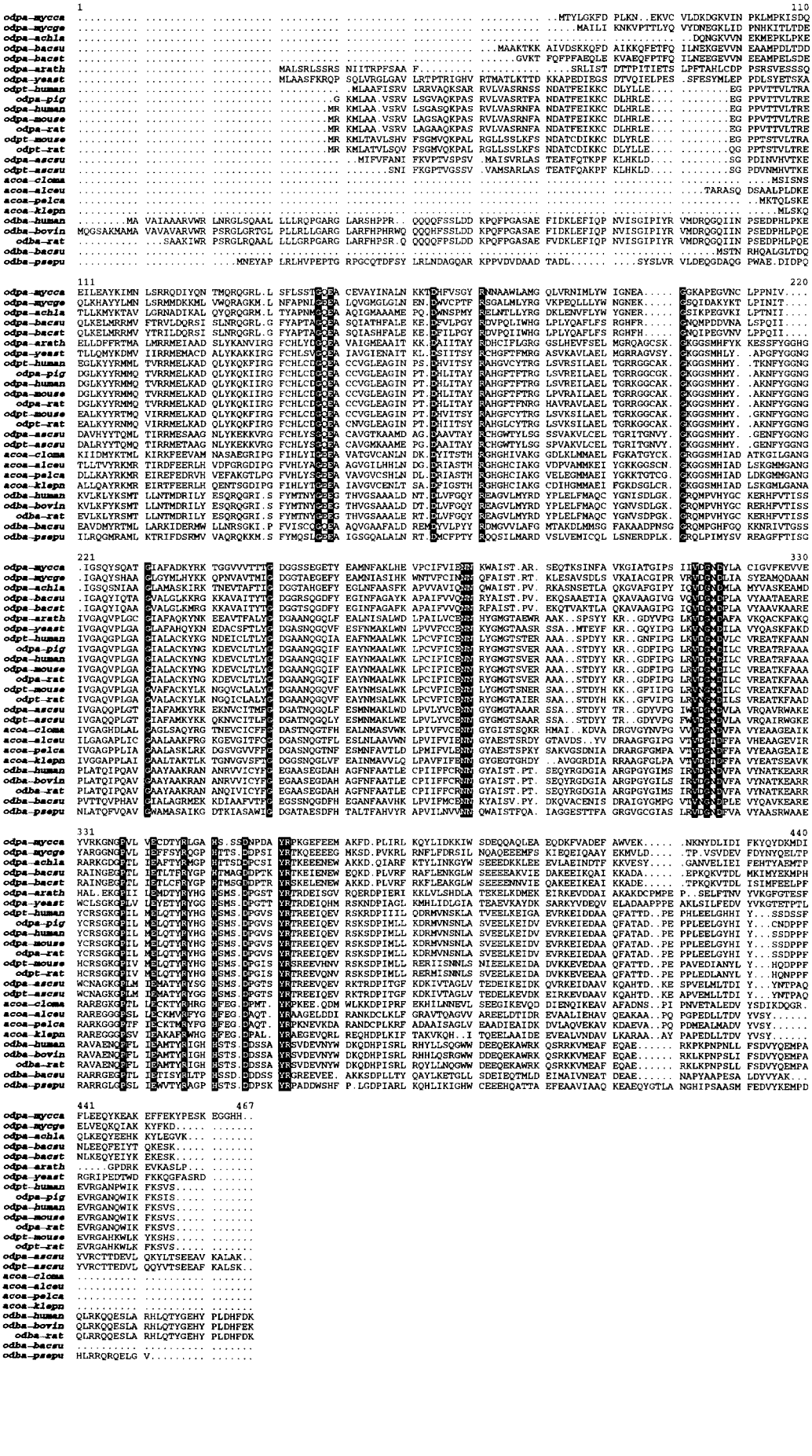


Fig. 6. Phylogenetic tree of sequenced proteins of the Enzyme I-α subunit family. Relative evolutionary distances are shown on the horizontal scale. Abbreviations are as in the legend to Figure 5.



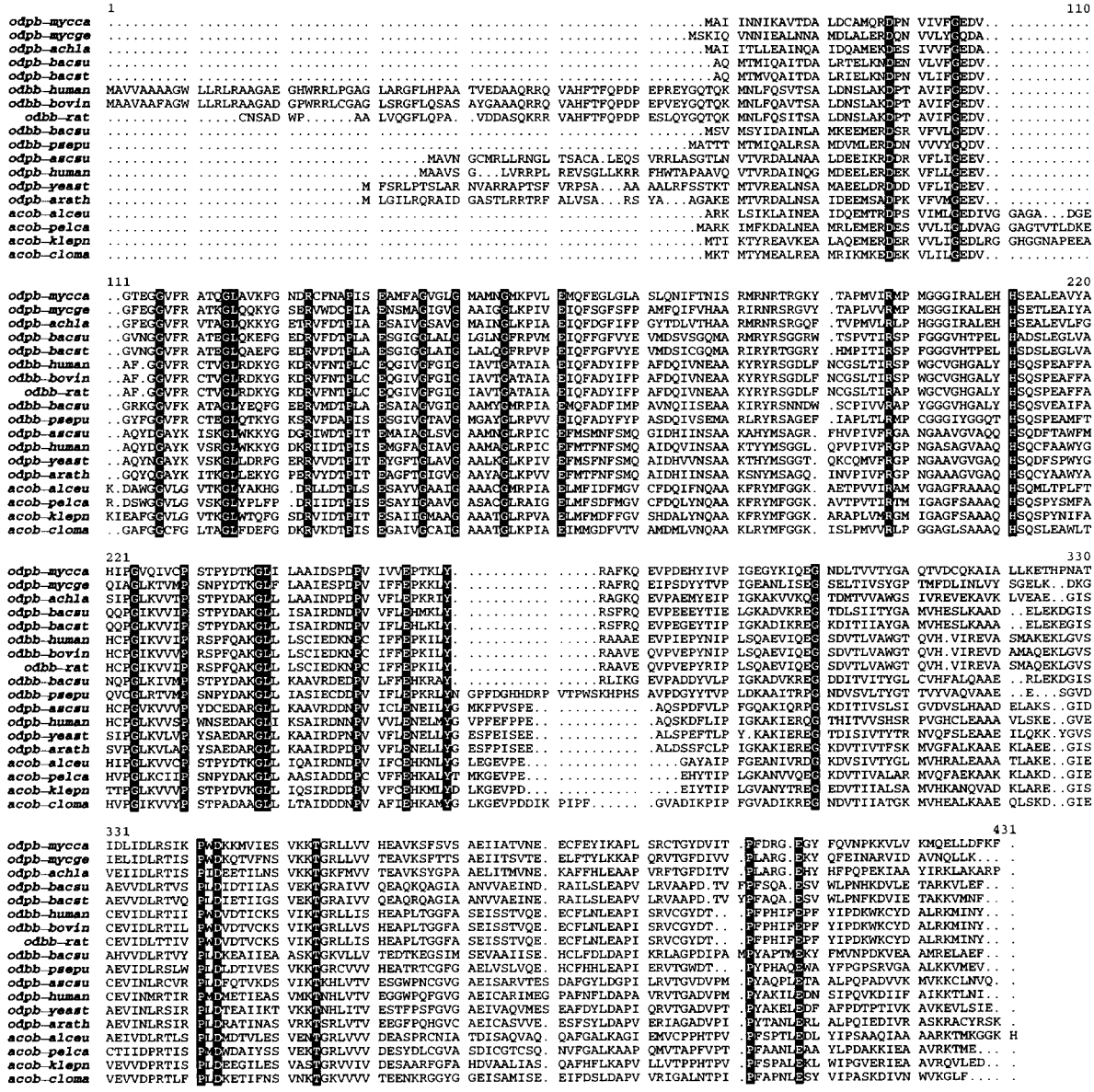


Fig. 7. Alignment of sequences of members of the Enzyme I-β subunit family. Numbering above the aligned sequences corresponds only to the residue in the alignment rather than to a residue number in any of the aligned proteins. Residues that are conserved in the listed Enzyme I-β proteins are shown in reverse shading. Abbreviations used and references to published sequences are: *M. capricolum* (odpb-mycca) (this work); *M. genitalium* (odpb-mycge) (MG273 from the TIGR database); *A. laidlawii* (odpb-achla) (Wallbrandt et al., 1992); *B. subtilis* (odpb-bacsu) (Hemila et al., 1990) and (odbb-bacsu) (Wang et al., 1993); *B. stearothermophilus* (odpb-bacst) (Borges et al., 1990), human (odbb-human) (Nobukuni et al., 1990a) and (odpb-human) (Ho & Patel, 1990); bovine (odbb-bovin) (Nobukuni et al., 1990b); rat (odbb-rat) (Zhao et al., 1992); *P. putida* (odbb-psepu) (Burns et al., 1988); *A. suum* (odpb-ascu) (Wheelock et al., 1991); yeast (odpb-yeast) (Miran et al., 1993); *A. thaliana* (odpb-arath) (Luethy et al., 1994); *A. eutrophus* (acob-alceu) (Priefert et al., 1991); *P. carbinolicus* (acob-pelca) (Oppermann & Steinbuchel, 1994); *K. pneumoniae* (acob-klepn) (Deng et al., 1994); *C. magnum* (acob-cloma) (Kruger et al., 1994). odpb, odbb, and acob refer to the genes encoding the β-subunits of Enzyme I of the pyruvate dehydrogenase, 2-oxoisovalerate dehydrogenase, and acetoin catabolism complexes.

respond to the lipoyl domains (approximately 70 residues). The proteins from *E. coli* and *Azotobacter vinelandii* contain three such domains; those from *E. faecalis*, *A. laidlawii*, *Alcaligenes eutrophus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Dictyostelium discoideum*, *Arabidopsis thaliana*, and human contain two; and all the others shown, including the *Mycoplasma* sequences, contain one lipoyl domain. Each one of the lipoyl domains contains a conserved lysine residue (the site of lipoylation), generally preceded by an aspartyl residue. In the case of *A. vinelandii*, the aspartate of the first lipoyl domain is replaced by alanine. For *Klebsiella pneumoniae*, the conserved aspartate is replaced by serine. All the lipoyl domains are also character-

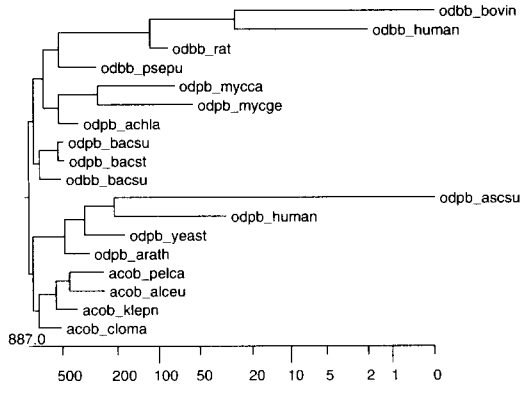


Fig. 8. Phylogenetic tree of sequenced proteins of the Enzyme I-β subunit family. Relative evolutionary distances are shown on the numerical scale. Abbreviations are as in the legend to Figure 7.

ized by the presence of a conserved GD pair. In the case of odp2 from *B. subtilis*, the G is replaced by N. For odo2 of *A. vinelandii*, odp2 of rat, and odp2 of human, the D is replaced by E. In approximately half of the proteins (odb2-psepu, odo2-azovi, odp2-rat, odo2-rat, odo2-human, odp2-entfa, odpa-achla, odp2-alceu, odp2-haein, odp2-pseae, odp2-dicdi, odp2-arath, odp2-human, odp2-ecoli, and odp2-azovi), the lipoyl domain is separated from the remainder of the protein by an A,P-rich linker. It is worth noting that the linker in odp2-dicdi is enriched in serine. The central portions of the proteins contain an E3 binding domain of approximately 50 residues and the carboxyl-terminal 250 residues corresponds to the catalytic domain, which effects the acyl transfer reaction. The conserved histidine (at residue 753) acts as the general base catalyst (Mattevi et al., 1992). A phylogenetic tree (Fig. 10) shows the preservation of the evolutionary relationship of the proteins from *M. capricolum*, *M. genitalium*, and *A. laidlawii*.

Following a gap of 18 bases, an open reading frame (from bases 4796 to 6686) was found; it corresponds to the gene encoding dihydrolipoamide dehydrogenase. The alignment shown in Figure 11 emphasizes a unique feature of the dihydrolipoamide dehydrogenase from *M. capricolum*. This protein contains an aminoterminal lipoyl domain (shown as a boxed grey area), a feature observed previously only in the DLDH proteins from *A. eutrophus* (Hein & Steinbuchel, 1994) and *Clostridium magnum* (Kruger et al., 1994). These lipoyl domains contains the characteristic conserved lysine residue (the site of lipoylation) (Mattevi et al., 1992), preceded by an aspartyl residue (residues 42 and 43). There are a number of totally conserved residues (shown in reverse shading) characteristic of dihydrolipoamide dehydrogenase sequences. The region from residues 171 to 180 corresponds to the motif GXGXXGYXXA, which is a possible nucleotide binding site. The conserved region from residues 206 to 218 corresponds to the signature GGXCLNXGCXP(S/T)K. In this region, the combination of the flavin ring with an adjacent disulphide bridge forms the redox center, which is involved with the transfer of the reducing equivalents from dihydrolipoamide to NAD^+. The vicinal cysteines in this region may bind lipoic acid and undergo reversible oxidation-reduction. The active species (dimeric) of E3 has four domains: FAD binding, NAD binding, central, and interface (Mattevi et al., 1992). A phylogenetic tree (Fig. 12) documents the consistency of the relationship of the *M. capricolum*, *M. genitalium*, and *A. laidlawii* sequences.

A space of 21 bases separates the coding sequence for dihydrolipoamide dehydrogenase from the next open reading frame. The region from bases 6707 to 7675 corresponds to the gene encoding phosphotransacetylase. The seven known sequences for phosphotransacetylase are aligned in Figure 13. The proteins from *E. coli* and *H. influenzae* are unique in that they have amino-terminal extensions of approximately 400 residues. All of the sequences show characteristic regions of total conservation, highlighted by reverse shading. A phylogenetic tree (Fig. 14) shows clearly a distinct branch for the proteins from *H. influenzae* and *E. coli* and a separate branch for the proteins from *M. genitalium* and *M. capricolum*.

A 13-base spacer separates the *pta* gene from the next open reading frame, encoding acetate kinase (from bases 7689 to 8866). The alignment shown in Figure 15 compares the six known acetate kinase sequences. The amino-terminal regions are characterized by a highly conserved sequence (residues 12–17, shown in reverse shading), GSSS(I/L)K, that may be involved in nucleotide binding. The phylogenetic tree shown in Figure 16 demonstrates the sequence relatedness of the *M. capricolum* and *M. genitalium* proteins, as well as the *E. coli*, *H. influenzae* similarities. Seventy-one bases separate the end of the sequence encoding acetate kinase from the one encoding *orfA*.

The sequence shown in Figure 2 was examined for regulatory features. Upstream of the *lplA* gene, within the coding sequence of *naox*, there is a possible −35 (TTGACA, bases 180–185, underlined) and −10 (TTTAAT, bases 204–209, boxed) promoter sequence. Upstream of the *odpA* gene, within the *lplA* coding sequence, there is a possible −35 (TTGACT, bases 1226–1231, underlined) and −10 (AAGAAT, bases 1249–1254, boxed) sequence. Upstream of *odpB*, within the *odpA* coding sequence, is found the potential −35 (TTGACA, bases 2328–2333, underlined) and −10 (CAAAAT, bases 2353–2358, boxed) sequence. Within the *odpB* coding sequence, upstream of *odp2*, potential −35 (TTGATA, bases 3350–3355, underlined) and −10 (TAAA AA, bases 3384–3389, boxed) sequences are located. Within the *dldH* coding region, upstream of *pta*, there are two potential −35 sequences (TTGGAA, bases 6523–6528 and TTGCTA, bases 6594–6599, underlined) and two potential −10 (AGAAAT, bases 6550–6555 and AACAAT, bases 6619–6624, boxed) sequences. Near the end of the *ack* coding sequence is located a potential −35 (TTGTTT, bases 8841–8846, underlined) and −10 (TTAAAT, bases 8864–8869, boxed) sequence. The spacer between the end of the *ack* gene and the beginning of *orfA* (71 bases) is AT-rich (marked with diverging arrows) and may form a stem-loop structure that plays a role in transcription termination of the *ack*-containing message.

Probable ribosome-binding sites are recognizable at appropriate positions before coding sequences. AGGAAAA (bases 270–276, shaded) is found before *lplA*; AGAAAGG (bases 1315–1321, shaded) is found before *odpA*; AGGAGGA (bases 2425–2431, shaded) is localized preceding *odpB*; AGAAAGG (bases 3447–3453, shaded) precedes *odp2*; AGTGAAG (bases 4781–4787, shaded) is localized in front of *dldH*; AGAAAGA (bases 6696–6702, shaded) precedes *pta*; AGGAGAA (bases 7675–7681, shaded) is found in front of *ack*; AGAAAGC (bases 8927–8933, shaded) is found in front of *orfA*.

A

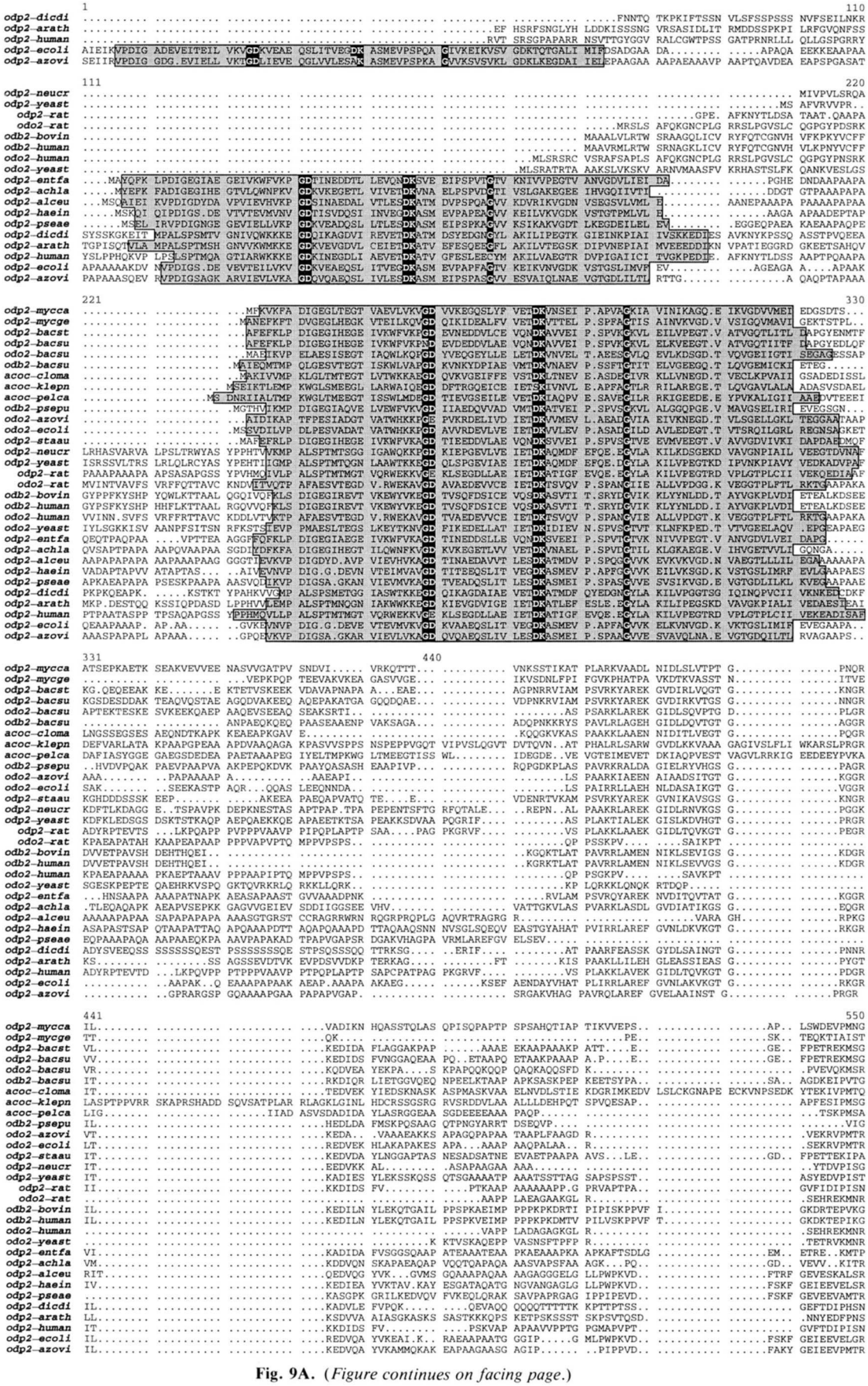


Fig. 9A. (*Figure continues on facing page.*)

B

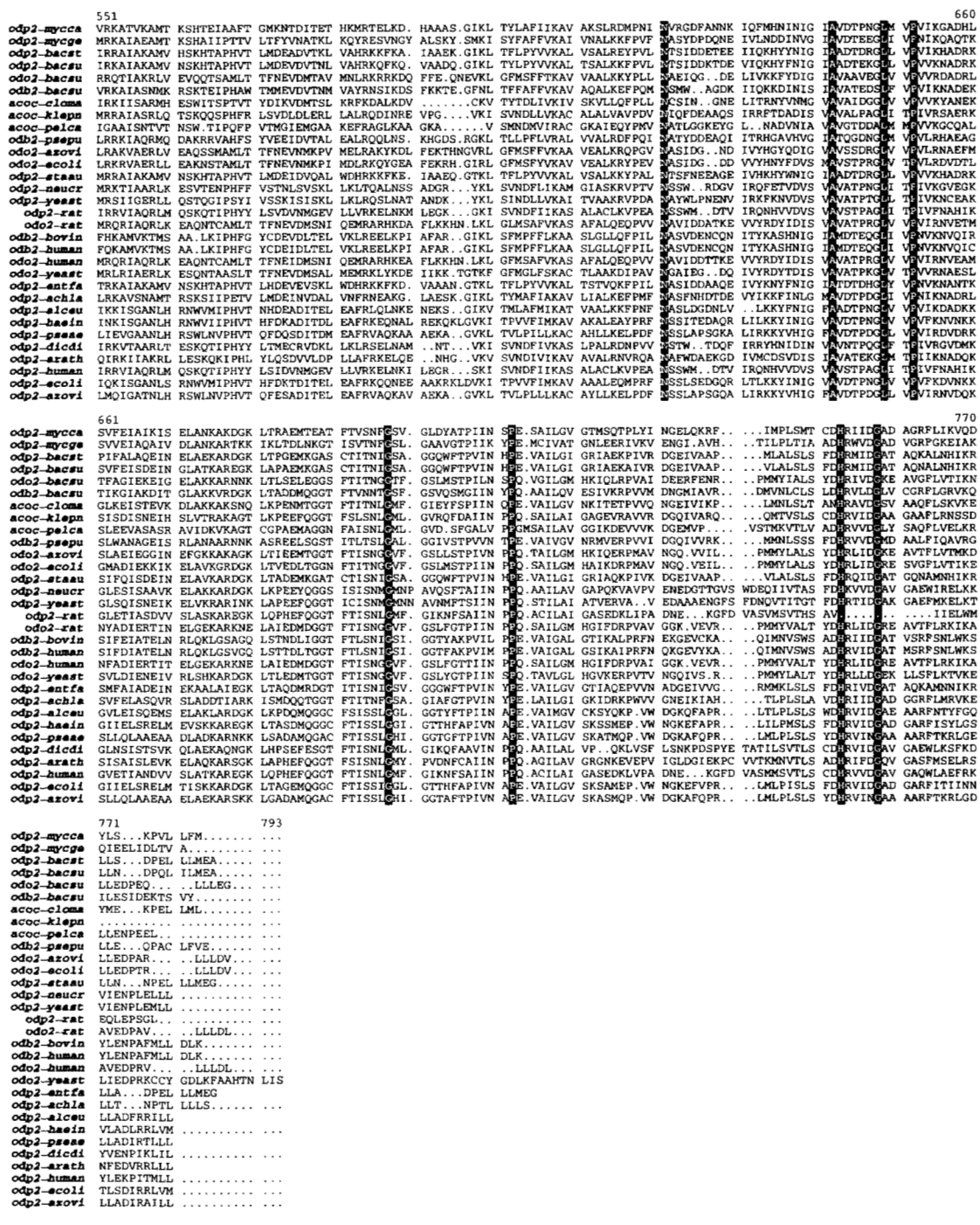


Fig. 9. Alignment of sequences of members of the Enzyme II family. Numbering above the aligned sequences corresponds only to the residue in the alignment rather than to a residue number in any of the aligned proteins. Residues that are conserved in the listed Enzyme II proteins are shown in reverse shading. Boxed shaded regions correspond to lipoyl domains. Abbreviations used and references to published sequences are: *M. capricolum* (odp2-mycca) (this work); *M. genitalium* (odp2-mycge) (MG272 from the TIGR database); *B. stearothermophilus* (odp2-bacst) (Borges et al., 1990); *B. subtilis* (odp2-bacsu) (Wang et al., 1993), (odb2-bacsu) (Wang et al., 1993), and (odo2-bacsu) (Carlsson & Hederstedt, 1989); *C. magnum* (acoc-cloma) (Kruger et al., 1994); *K. pneumoniae* (acoc-klepn) (Deg et al., 1994); *P. carbinolicus* (acoc-pelca) (Oppermann & Steinbuchel, 1994); *P. putida* (odb2-psepu) (Burns et al., 1988); *A. vinelandii* (odo2-azovi) (Westphal & de Kok, 1990) and (odp2-azovi) (Hanemaaijer et al., 1988); *E. coli* (odo2-ecoli) (Spencer et al., 1984) and (odp2-ecoli) (Guest, 1987); *Staphylococcus aureus* (odp2-staur) (Hemila, 1991); *Neurospora crassa* (odp2-neucr) (Kreader et al., 1989); yeast (odp2-yeast) (Niu et al., 1988) and (odo2-yeast) (Repetto & Tzagoloff, 1990); rat (odp2-rat) (Gershwin et al., 1987) and (odo2-rat) (Nakano et al., 1991); bovine (odb2-bovin) (Lau et al., 1988); human (odb2-human) (Lau et al., 1992), (odp2-human) (Thekkumkara et al., 1988), and (odo2-human) (Nakano et al., 1993); *E. faecalis* (odp2-entfa) (Allen & Perham, 1991); *A. laidlawii* (odp2-achla) (Wallbrandt et al., 1992); *A. eutrophus* (odp2-alceu) (Hein & Steinbuchel, 1994); *H. influenzae* (odp2-haein) (HI232 from the TIGR database); *P. aeruginosa* (odp2-pseae) (Genbank accession no. U47920); *D. discoideum* (odp2-dicdi) (Genbank accession no. u06634); *A. thaliana* (odp2-arath) (Guan et al., 1995). odp2, odb2, and odo2 refer to the genes encoding the Enzyme IIs of the pyruvate dehydrogenase; α-oxo acid dehydrogenase and 2-oxoglutarate dehydrogenase complexes, respectively; and acoc refers to acetoin dehydrogenase EII.

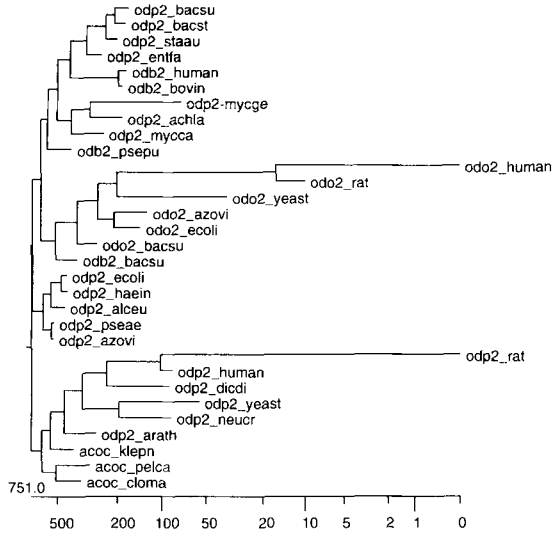


Fig. 10. Phylogenetic tree of sequenced proteins of the Enzyme II family. Relative evolutionary distances are shown on the numerical scale. Abbreviations are as in the legend to Figure 9.

Gene transcription analyses

The cloned sequence encodes 13 genes that might be transcribed in the same direction (see Fig. 1). The only significant gap between sequences occurs between the *ack* and *orfA* open reading frames. The program TERMINATOR was used to search the cloned sequence for potential transcription termination sequences (designated by diverging arrows in Fig. 2). Nineteen bases downstream of the translation termination codon for *ack*, there is a possible transcription termination region (from nucleotides 8886–8914). Using the program FOLDRNA, the stability of this stem-loop structure was calculated to be −11.8 kcal/mol. The stem-loop structure formed contains a perfectly matched stem 12 bases long. It is therefore possible that the region from *naox* to *ack* is transcribed as a single mRNA. Evidence was presented previously (Zhu et al., 1994) that the *ptsI* and *crr* genes constituted an operon. Consequently, it may be the case that the *orfA* and *kdtB* genes are cotranscribed.

Discussion

The sequence analysis presented here (see Figs. 1, 2) demonstrates the presence in *M. capricolum* of a unique arrangement of genes involved in the metabolism of pyruvate. In other bacterial species where gene mapping has been performed (*E. coli*, *H. influenzae*, *M. genitalium*), the genes are somewhat scattered throughout the genome. For example, in *E. coli*, the genes encoding the pyruvate dehydrogenase complex are located at 3 min on the genetic map, whereas the *ack*, *pta* and *ptsH*, *ptsI* and *crr* genes are in the 50–52-min region. In contrast, in *M. capricolum*, all the relevant genes are clustered in a single region. The positioning of these genes close to the *ptsI-crr* operon (whose products use PEP to form pyruvate) may also be of some regulatory significance. The scheme shown in Figure 17 indicates that all the enzymes required for the conversion of PEP to acetate and ATP, including those activities used for lipoylation of the pyruvate dehydrogenase and regeneration of NAD from NADH, are accounted for in the region of the *M. capricolum* genome sequenced in this study (see Fig. 1).

We reported previously (Zhu et al., 1993) that the HPr protein from *M. capricolum* has an unusually high isoelectric point. Using the computer program PEPTIDESORT, we calculated the pIs of the protein sequences deduced in this study. The pIs of the EI and EII proteins of the pyruvate dehydrogenase complex were in the range 5.3–6.75 and were similar for both *M. capricolum* and *M. genitalium*. However, several of the proteins from *M. genitalium* showed significantly higher pIs than the corresponding proteins from *M. capricolum*. For DLDH, the pIs for *M. capricolum* and *M. genitalium* were 5.35 and 7.08, respectively. For lipoate-protein ligase, the pIs for *M. capricolum* and *M. genitalium* were 6.97 and 9.29, respectively. For phosphotransacetylase, the pIs for *M. capricolum* and *M. genitalium* were 5.24 and 7.48, respectively. For acetate kinase, the pIs for *M. capricolum* and *M. genitalium* were 6.67 and 9.09, respectively. The significance of the widespread occurrence of proteins with high isoelectric points in *M. genitalium* remains to be clarified.

In order to evaluate the possibility that the genes encoding the enzymes involved in pyruvate metabolism are part of a polycistronic operon(s), northern blotting and primer extension experiments using probes derived from the plasmids described in Figure 1 were performed. No detectable mRNA species were found. Similar lack of success in detecting *ack*- or *pta*-specific mRNA species was reported for *Methanosarcina thermophila* (Latimer & Ferry, 1993) and it was suggested that the mRNAs may have a short half-life or be degraded rapidly during the RNA preparation. Consequently, the nature of the transcripts encoding the enzymes for pyruvate metabolism remains to be established.

The dihydrolipoamide dehydrogenase from *M. capricolum* is atypical, containing an amino-terminal lipoyl domain (see Fig. 11). This structure is also shared by the enzymes from *A. eutrophus* and *C. magnum*. This observation opens the possibility that effective function of the pyruvate dehydrogenase complex may be possible with the association of lipoate residues with either the E2 or E3 components. It is interesting to note that a recent description of an outer membrane protein from *Neisseria meningitidis* (de la Sierra et al., 1994) indicated that it contained an amino-terminal lipoyl domain and was otherwise homologous to lipoamide dehydrogenases.

The *M. capricolum* pyruvate dehydrogenase and *C. magnum* acetoin dehydrogenase complexes show a unique similarity. Each of these complexes contain an E2 with a single lipoyl domain, as well as an E3 with a single lipoyl domain. Comparison of the sequences of these lipoyl domains (see Figs. 9, 11) show that they are almost exact duplicates. This suggests that the lipoyl domains in the E3 proteins of these organisms arose by a duplication of the preexisting domain in the genes encoding the E2s.

In contrast, the pyruvate dehydrogenase complex of *A. eutrophus* is characterized by an E2 with two lipoyl domains, as well as an E3 with a single lipoyl domain. In this case, the two lipoyl domains of E2 are essentially identical, consistent with the idea that they arose by a duplication mechanism. However, the single lipoyl domain in the E3 diverges considerably from the sequences in the E2. Therefore, it seems unlikely that the lipoyl domain of the E2 in this organism arose by a simple duplication of the sequences in the gene encoding the E2.

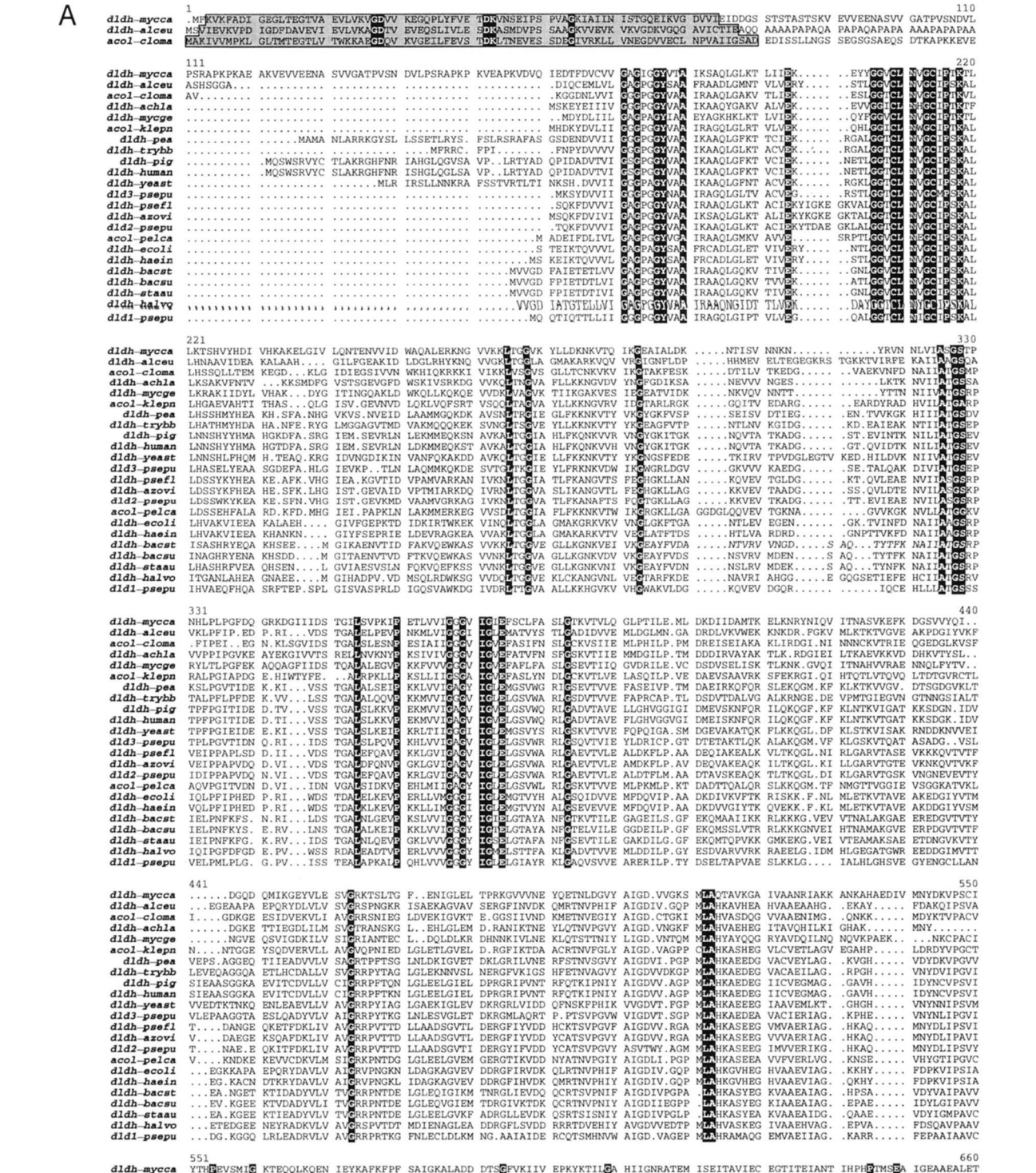


Fig. 11A. (*Figure continues on following page.*)

B

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                661              678
dldh-mycca      GKAIHF.... ........
dldh-alceu      GTCTDVPPPR KR......
acol-cloma      NQAIHMMNK. ........
dldh-achla      .......... ........
dldh-mycge      DYPS..... ........
acol-klepn      DQPLHQ.... ........
  dldh-pea      DKPIHI.... ........
dldh-trybb      AKTINF.... ........
  dldh-pig      GKAINF.... ........
dldh-human      GKSINF.... ........
dldh-yeast      DKAIHC.... ........
dld3-psepu      GMAMQI.... ........
dldh-psefl      GHAIHIANRK KR......
dldh-azovi      GHAIHVANRK K.......
dld2-psepu      GGAIHVANRK KR......
acol-pelca      GAAVHC.... ........
dldh-ecoli      GSITDLPNPK AKKK....
dldh-haein      GSITDLPNAK AKEKIISI
dldh-bacst      GTPIHIITK. ........
dldh-bacsu      GSPIHIVK.. ........
dldh-staau      GYPIHTM... ........
dldh-halvo      GQAIHTLNR. ........
dld1-psepu      GHALHI.... ........
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Fig. 11. Alignment of sequences of members of the dihydrolipoamide dehydrogenase family. Numbering above the aligned sequences corresponds only to the residue in the alignment rather than to a residue number in any of the aligned proteins. Residues that are conserved in the listed Enzyme II proteins are shown in reverse shading. Boxed shaded region corresponds to a lipoyl domain. Abbreviations used and references to published sequences are: *M. capricolum* (dldh-mycca) (this work); *A. eutrophus* (dldh-alceu) (Hein & Steinbuchel, 1994); *C. magnum* (acol-cloma) (Kruger et al., 1994); *A. laidlawii* (dldh-achla) (Wallbrandt et al., 1992); *M. genitalium* (dldh-mycge) (MG271 from the TIGR database); *K. pneumoniae* (acol-klepn) (Genbank accession no. U30887); pea (dldh-pea) (Bourguignon et al., 1992); *Trypanosoma brucei* (dldh-trybb) (Else et al., 1993); pig (dldh-pig) (Otulakowski & Robinson, 1987); human (dldh-human) (Pons et al., 1988); yeast (dldh-yeast) (Browning et al., 1988); *P. putida* (dld2-psepu) (Palmer et al., 1991a), (dld1-psepu) (Burns et al., 1989), and (dld3-psepu) (Palmer et al., 1991b); *Pseudomonas fluorescens* (dldh-psefl) (Benen et al., 1989); *A. vinelandii* (dldh-azovi) (Westphal & de Kok, 1988); *P. carbinolicus* (acol-pelca) (Oppermann & Steinbuchel, 1994); *E. coli* (dldh-ecoli) (Guest, 1987); *H. influenzae* (dldh-haein) (HI1231 frrom the TIGR database); *B. stearothermophilus* (dldh-bacst) (Borges et al., 1990); *B. subtilis* (dldh-bacsu) (Hemila et al., 1990); *S. aureus* (dldh-staau) (Hemila, 1991); *Haloferax volcanii*, (dldh-halvo) (Vettakkorumakankav & Stevenson, 1992). dldh, dld1, and dld2 refer to the genes encoding dihydrolipoamide dehydrogenases from the pyruvate, 2-oxoglutarate, and branched chain α-ketoacid dehydrogenase complexes, respectively. dld3 refers to the third dehydrogenase isolated from *P. putida* and acol refers to the dihydrolipoamide dehydrogenase of the acetoin dehydrogenase complex.

Mycoplasmas are generally believed to be descendants of Gram-positive bacteria. All the *M. capricolum* proteins involved with the metabolism of pyruvate described here show phylogenetic relatedness to the homologous proteins from Gram-positive bacteria and *M. genitalium*.

The complete genome of *M. genitalium* has been reported recently (Fraser et al., 1995). Because it might be expected that these two species are closely related, the question was posed concerning the genomic locations of the genes encoding enzymes of pyruvate metabolism. Figure 18 shows a comparison of the location of the genes of interest in *M. capricolum* and *M. genitalium*. It is most surprising to see that, whereas all the genes involved with pyruvate metabolism are clustered in *M. capricolum*, this is not the case in *M. genitalium*. In both organisms, the *ptsI* genes are located approximately 500 kb from the replication origin. Further, the *gyrA,B* complex is separated from the *naox*, *odpA*, *odpB*, *odp2*, *dldH*, *lplA* complex by approximately 300 kb in both organisms. Clearly, there have been extensive rearrangements in the genomes of these organisms during evolution, resulting in resolution of *gyrA,B* from the replication origin in the case of *M. capricolum* and scattering of genes involved with pyruvate metabolism in *M. genitalium*.

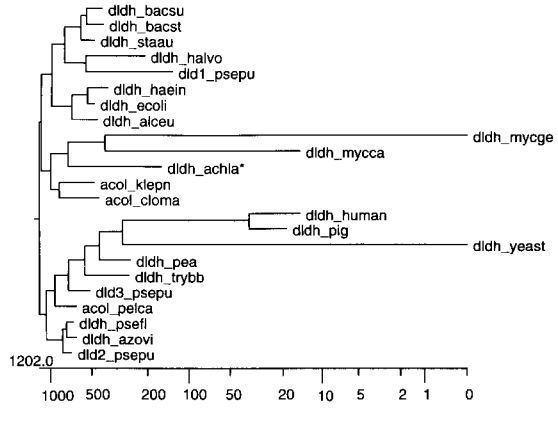

Fig. 12. Phylogenetic tree of sequenced proteins of the dihydrolipoamide dehydrogenase family. Relative evolutionary distances are shown on the numerical scale. Abbreviations are as in the legend to Figure 11. *, corresponds to a partial amino acid sequence (see Fig. 11).

In summary, the present work has demonstrated the unique arrangement of the genes encoding enzymes involved with pyruvate metabolism in *M. capricolum* and described some unique properties of the products of these genes.

Materials and methods

Growth of cells

M. capricolum (kid strain) were grown in modified Edwards medium at pH 8, as described previously (Mugharbil & Cirillo, 1978). After harvesting, cells were stored as frozen pellets for future use.

Nucleic acid preparations

DNA was prepared from frozen cells as previously described (Ausubel et al., 1990). RNA was prepared by suspension of cells (100 mg) in buffer (1 mL) containing 50 mM Tris-HCl, pH 6.8, 2 mM EDTA, and 1% SDS; the suspension was mixed with 5 mL of 4 M guanidium thiocyanate homogenization buffer

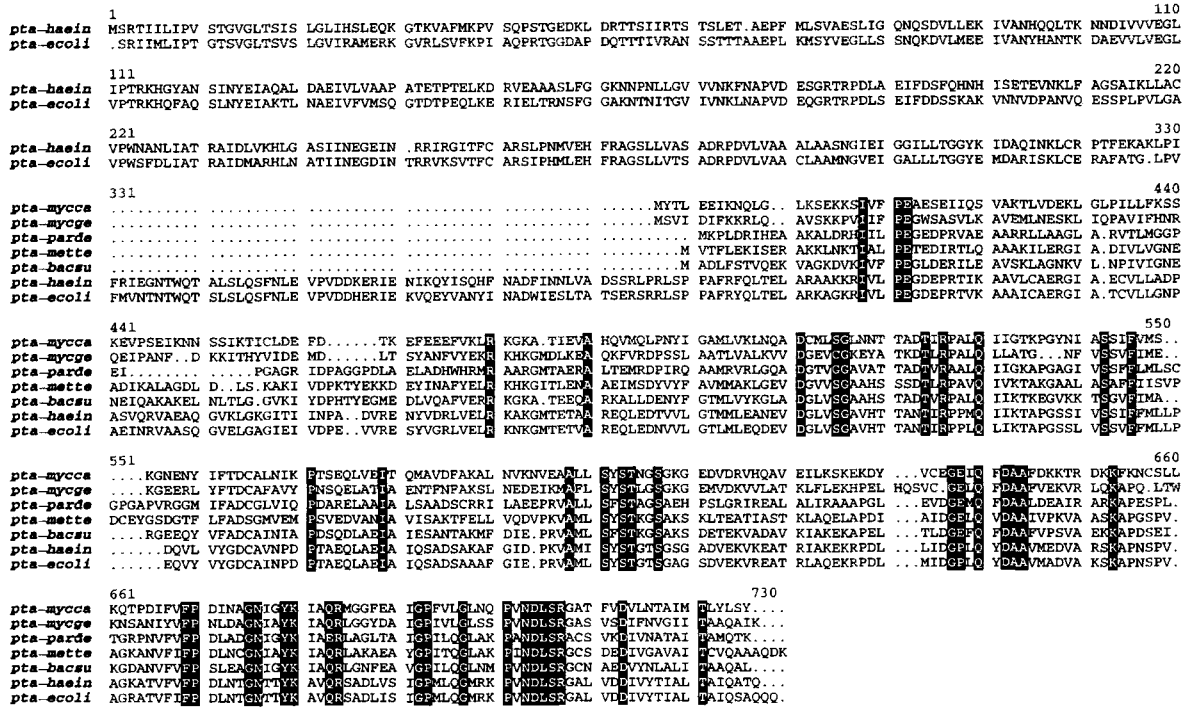

Fig. 13. Alignment of sequences of members of the phosphotransacetylase family. Numbering above the aligned sequences corresponds only to the residue in the alignment rather than to a residue number in any of the aligned proteins. Residues that are conserved in the listed phosphotransacetylase proteins are shown in reverse shading. Abbreviations used and references to published sequences are: *M. capricolum* (pta-mycca) (this work); *M. genitalium* (pta-mycge) (MG299 from the TIGR database); *Paracoccus denitrificans* (pta-parde) (Van Spanning et al., 1995); *M. thermophila* (pta-mette) (Latimer & Ferry, 1993); *B. subtilis* (pta-bacsu) (Glaser et al., 1993); *H. influenzae* (pta-haein) (H11203 from the TIGR database); *E. coli* (pta-ecoli) (Kakuda et al., 1994).

(Sambrook et al., 1989). The cell suspension was frozen in dry ice-ethanol, then warmed briefly in a 64 °C water bath (Salser et al., 1967). Total RNA was purified by ultracentrifugation through 5.7 M CsCl/10 mM EDTA (Sambrook et al., 1989).

Cloning and screening

Genomic DNA fragments produced by digestion of *M. capricolum* DNA with *Hind* III (9,542-bp fragment), *Xba* I (6,654-bp fragment), or *Spe* I (4,384-bp fragment) were cloned into the vector pBluescript II KS$^+$ (pKSII$^+$). Recombinant plasmids were used to transform Epicurean coli XLI-Blue Competent cells (Stratagene). Colonies were lifted onto nylon membranes (NEN Research Products, NEF-978). [^{32}P]5′-end-labeled oligonucleotide probes (1×10^6 cpm/mL of hybridization solution) were used for selecting positive clones. Oligonucleotide probes, synthesized as trityl-off derivatives on an Applied Biosystems 380B DNA synthesizer, were labeled with [γ^{32}P]ATP by the DNA 5′-end-labeling method (Sambrook et al., 1989). Prehybridization was performed at 40 °C for 4 h in 6× SSPE/0.1% SDS/10× Denhardt's solution containing 20 mg/mL tRNA and 50 mg/mL of denatured heterologous DNA. Hybridization was performed at 42 °C for 16 h in 6× SSPE/10% SDS solution containing 1.4×10^6 cpm of [^{32}P]-labeled oligonucleotide/mL. The membrane was finally washed in 0.5× SSPE/1% SDS solution at 40 °C for 30 min. Positive clones were detected by autoradiography.

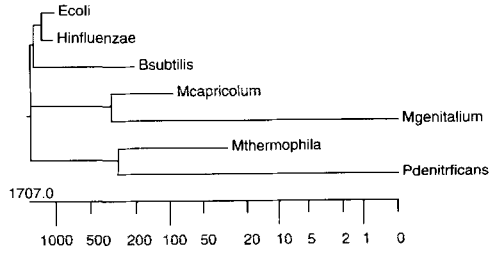

Fig. 14. Phylogenetic tree of sequenced proteins of the phosphotransacetylase family. Relative evolutionary distances are shown on the numerical scale. Abbreviations are as in the legend to Figure 13.

DNA sequencing

DNA sequencing on both strands of the DNA was performed by the dideoxy chain termination method of Sanger et al. (1977), with [α^{35}S]dATP, using Sequenase 2.0 (United States Biochemicals) DNA sequencing kits. M13 forward or reverse primers or

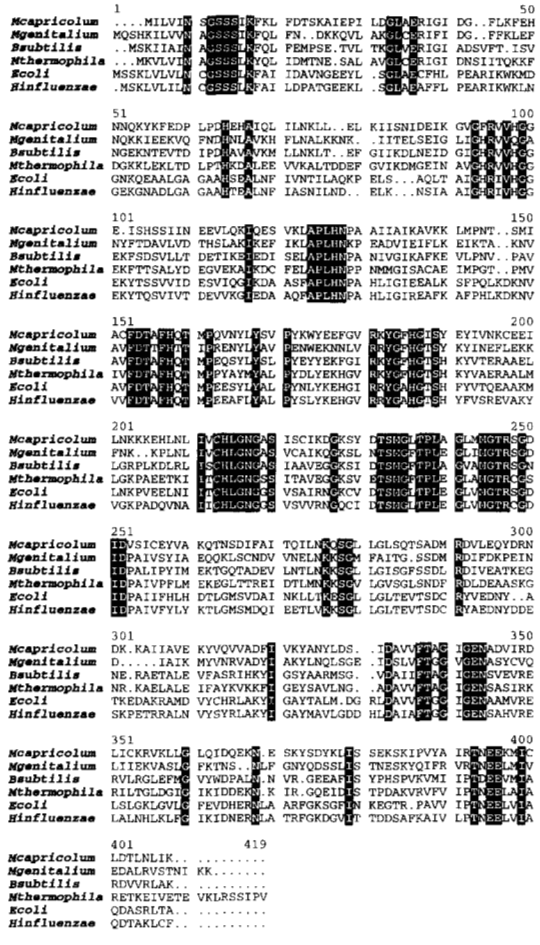

Fig. 15. Alignment of sequences of members of the acetate kinase family. Numbering above the aligned sequences corresponds only to the residue in the alignment rather than to a residue number in any of the aligned proteins. Residues that are conserved in the listed phosphotransacetylase proteins are shown in reverse shading. Abbreviations used and references to published sequences are: *M. capricolum* (Mcapricolum) (this work); *M. genitalium* (Mgenitalium) (MG357 from the TIGR database); *B. subtilis* (Bsubtilis) (Grundy et al., 1993); *M. thermophila* (Mthermophila) (Latimer & Ferry, 1993); *E. coli* (Matsuyama et al., 1989); *H. influenzae* (Hinfluenzae) (H11204 from the TIGR database).

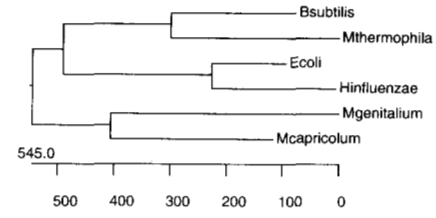

Fig. 16. Phylogenetic tree of sequenced proteins of the acetate kinase family. Relative evolutionary distances are shown on the numerical scale. Abbreviations are as in the legend to Figure 15.

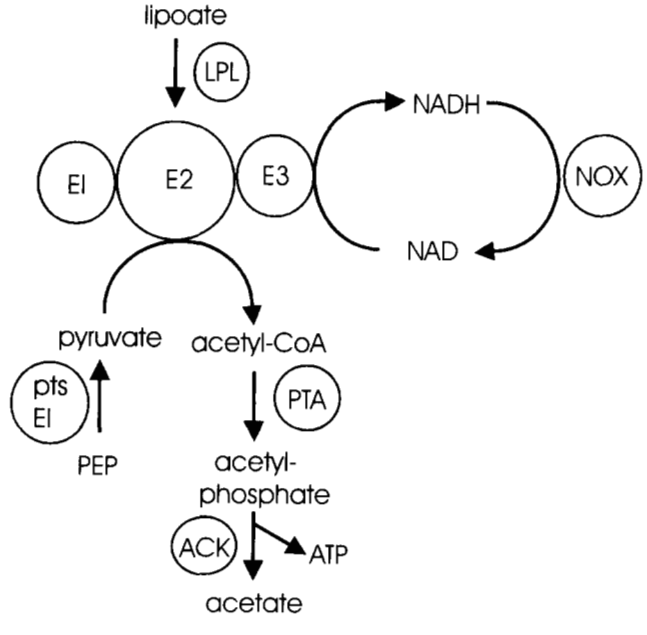

Fig. 17. Metabolic scheme for conversion of phosphoenolpyruvate to acetate in *M. capricolum*.

specific primers complementary to previously determined sequences were used.

Computer analyses

Analyses of DNA and protein sequence were performed using the GCG programs, version 7.2 (Devereux et al., 1984). Isoelectric points were calculated using the PEPTIDESORT program. A search for transcription termination sites used the TERMINATOR program. Stem-loop structures were analyzed using FOLDRNA. Translation frames were detected using the MAP program. Phylogenetic trees were constructed using the MEGALIGN module of the LaserGene program (DNAStar, Madison, Wisconsin) by the method of Hein (1990).

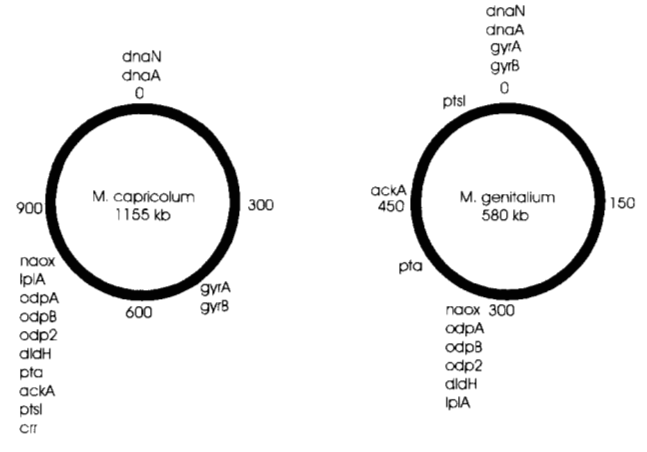

Fig. 18. Comparison of positions on the genetic map of various genes in *M. capricolum* and *M. genitalium*.

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