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Secretory S Complex of *Bacillus subtilis*: Sequence Analysis and Identity to Pyruvate Dehydrogenase

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We have cloned the operon coding for the *Bacillus subtilis* S complex, which has been proposed to be a component in protein secretion machinery. A lambda gt10 library of *B. subtilis* was screened with antiserum directed against the *Staphylococcus aureus* membrane-bound ribosome protein complex, which is homologous to the *B. subtilis* S complex. Two positive overlapping lambda clones were sequenced. The S-complex operon, 5 kilobases in size, was shown to contain four open reading frames and three putative promoters, which are located upstream of the first, the third, and the last gene. The four proteins encoded by the operon are 42, 36, 48, and 50 kilodaltons in size. All of these proteins were recognized by antisera separately raised against each protein of the *S. aureus* membrane-bound ribosome protein and *B. subtilis* S complexes, thus verifying the S-complex identity of the lambda clones. Sequence analysis revealed that all four proteins of the *B. subtilis* S complex are homologous to the four subunits of the human pyruvate dehydrogenase (PDH). Also, the N terminus of the 48-kilodalton protein was found to have 70% amino acid identity with the N-terminal 211 amino acids, determined so far, from the E2 subunit of *B. stearothermophilus* PDH. Furthermore, chromosomal mapping of the S-complex operon gave a linkage to a marker gene located close to the previously mapped *B. subtilis* PDH genes. Thus, the S complex is evidently identical to the *B. subtilis* PDH, which has been shown to contain four subunits with molecular weights very similar to those of the S complex. Therefore, we propose that the S complex is not a primary component of protein secretion.

Several secreted proteins of *Escherichia coli* and *Bacillus subtilis* are synthetized by membrane-bound ribosomes (55, 58). This led to the comparison of *B. subtilis* membrane fractions free of ribosomes (free membranes) with those with bound ribosomes (complexed membranes). Some of the proteins unique to complexed membranes were presumed to be components of the protein secretion machinery (33, 44). One of these proteins (64 kilodaltons [kDa]) appeared to be located between the membrane and the attached ribosomes, since it was protected against trypsin or proteinase K, unless the membrane fraction was first treated with EDTA, which detaches ribosomes (15, 34). Antiserum raised against the 64-kDa protein immunoprecipitated three additional proteins of 41, 36, and 60 kDa. This set of four proteins was termed the S complex (secretory complex). The 41-, 36-, and 64-kDa proteins appeared to have a stoichiometric ratio, while the 60-kDa protein was found in smaller amounts. The S complex was attached to membrane-free ribosomes, whereas the 64-kDa protein was also found in the cytosol and in the complexed membrane, without the other proteins. Based on these findings, it was suggested that the S complex plays a cyclic role in protein secretion, mediating initiation of the secretion process by promoting attachment of ribosomes to the membrane (15).

A similar rationale has independently led to the search for proteins attached to membrane-bound ribosomes in *Staphylococcus aureus*. Ribosomes released from *S. aureus* membranes were shown to bind a 60-kDa protein, which was not found in cytoplasmic ribosomes. Antiserum to this protein caused immunoprecipitation of four proteins with molecular weights of 46,000, 41,000, 71,000, and 60,000 (2). This set of four proteins was designated the membrane-bound ribosome protein (MBRP) complex. These four proteins were found in roughly stoichiometrical amounts in the complex, although the 60-kDa protein appeared to be loosely bound to it (2, 3). The MBRP complex was found both on membrane and in the cytoplasm. The membrane-bound fraction of the MBRP complex appeared to be mostly bound to ribosomes, since it was protected against trypsin (3). Thus, the MBRP complex seems to be shielded by ribosomes similarly to the *B. subtilis* 64-kDa protein. The efficient binding of MBRP to membrane-bound ribosomes was considered to support the assumption that the MBRP complex participates in protein secretion.

The MBRP complex was also shown to become more membrane attached under conditions enhancing protein secretion, even though the total amount of the complex remained quite constant (3). This redistribution supported the concept that MBRP participates in secretion. Moreover, antiserum to the *S. aureus* 60-kDa protein immunoprecipitated from *B. subtilis* cell lysate a set of four proteins of 43, 40, 64, and 62 kDa (1). The molecular weights of these proteins are similar enough to those of the *B. subtilis* S complex to indicate that the antiserum directed against the *S. aureus* MBRP complex recognizes the *B. subtilis* S complex.

No obvious counterpart for the S complex has been found in *E. coli*. This may suggest dissimilarity of protein translocation mechanisms in gram-positive and gram-negative bacteria, which has made the S complex even more intriguing. However, data supporting the participation of the S complex in protein translocation are indirect and nonconclusive. More definitive elucidation of the function of the MBRP and S complexes has suffered from the lack of genetic analysis and of usable in vitro translocation systems in *B. subtilis* and

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S. aureus. The *S. aureus* MBRP complex operon has recently been cloned, but not yet sequenced (4).

In this paper, we describe the cloning and sequencing of the *B. subtilis* S-complex operon. The sequence analysis and chromosomal mapping data strongly suggest that the S complex is identical to the pyruvate dehydrogenase (PDH) complex, which connects the glycolysis to the tricarboxylic acid cycle. This result implicates that the S complex has no major role in protein secretion.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and growth media. *B. subtilis* 168 strains were BRB1 (*sacA321 metB5*) and BRB34 (*his sacA321*) (our collection). *B. subtilis* QB943 (*pyrD1 ilvA1 thyA1 thyB1 trpC2*), the kit 4 reference strain (16), was used for genetic mapping with phage PBS1. The *E. coli* cloning host for plasmids and bacteriophage M13 derivatives was TG1 [K-12 Δ(*lac-pro*) *supE thi hsdD5*(F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*-Δ-M15)]. *E. coli* NM514 and L87 strains for lambda gt10 were included in the Amersham cDNA cloning kit as insert selection (*hfl*$^+$) and control strain, respectively. Strains were grown in Luria broth or L agar (42), unless otherwise indicated. QB943 was grown on Penassay medium (Difco Laboratories) supplemented with 20 μg of thymine per ml unless otherwise stated. Bacteria carrying antibiotic resistance markers were grown in the presence of the appropriate antibiotic in concentrations of 100 μg of ampicillin or 5 μg of chloramphenicol per ml. SMS medium (5) supplemented with 0.1% solution A was used in cell cultivation for immunoprecipitation. Solution A contained 120 g of $MgCl_2$, 0.5 g of $FeSO_4$, 0.5 g of $ZnSO_4$, 70 mg of $MnSO_4$, 6 mg of $CuSO_4$, and 2 mg of $K_2Cr_2O_7$ per liter.

Genetic and DNA techniques. Transformation of *E. coli* and *B. subtilis* was by the methods of Hanahan (25) and Gryczan et al. (23), respectively. M13 phage infections and propagations in TG1 cells were as described earlier (45). Lambda infection was by standard methods (42). Transduction with PBS1 was essentially as described by Young and Wilson (71). DNA manipulations were performed by standard methods (42). DNA probes for Southern (59) and Northern hybridization experiments were labeled by nick translation with [α-^{32}P]dCTP (>3,000 Ci/mmol; Amersham), using the protocol of the Boehringer-Mannheim nick translation kit. Plasmid DNAs were prepared essentially by the alkaline method (6). The oligonucleotides were made with an Applied Biosystems synthesizer, model 381A. For mRNA analysis, total RNA was isolated from *B. subtilis* BRB1 cells at late stationary phase followed by agarose gel electrophoresis and Northern (RNA) blotting as described before (52). mRNAs of the S-complex operon were detected by nick-translated pKTH1878 and pKTH1879 probes and visualized by autoradiography.

Reagents and antisera. Reagents and enzymes were obtained from commercial suppliers. The specific rabbit antisera separately raised against the four individual proteins of the *S. aureus* MBRP complex and the antiserum directed against the *S. aureus* 60- and 70-kDa MBRP proteins have been described earlier (3, 4). Purified immunoglobulin G fractions of rabbit antisera to each protein of the *B. subtilis* S complex were kindly donated by P. C. Tai.

***B. subtilis* gene library.** Chromosomal DNA was isolated from *B. subtilis* BRB1 by the method of Marmur (43). A *B. subtilis* gene library was constructed into lambda gt10 (36), utilizing a cDNA cloning kit (Amersham). Chromosomal DNA of BRB1 was partially digested with *Hae*III and separated by agarose gel electrophoresis followed by isolation and purification of the size fraction of approximately 7 kilobases (kb). These DNA fragments were treated with *Eco*RI methylase and ligated with *Eco*RI linkers. After ligation, the fragments were digested with *Eco*RI, purified from the agarose gel, religated with lambda gt10 arms, and packaged into lambda particles. The library contained approximately 5×10^7 PFU/ml, and the insertion frequency was roughly 90% as determined by the difference in the infection frequencies between the lambda selective (NM514) and nonselective (L87) strains. To detect positive clones, plaques were blotted to nitrocellulose filters (Schleicher & Schuell) (68) and treated with antiserum raised against the *S. aureus* 60- and 70-kDa MBRP proteins. Filters were stained with anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Protoblot; Promega).

Immunoprecipitation of the S complex. *B. subtilis* BRB34 was grown at 37°C in SMS-A medium supplemented with 1% glucose and 20 μg of each amino acid per ml, except Met, Trp, Gln, and Asn. A 1-ml portion of cells grown to an A_{660} of 0.5 was labeled with 30 μCi of [^{35}S]methionine (>1,000 Ci/mmol; Amersham) by shaking for 30 min at 37°C. After labeling, cells were centrifuged, suspended in 1 ml of SMM (0.5 M sucrose, 20 mM $MgCl_2$, 20 mM maleate, pH 7.5), and divided into aliquots and frozen at −20°C. A 250-μl amount of frozen cells was treated with 200 μg of lysozyme, 20 μg of RNase A, and 10 μg of DNase I for 5 min at room temperature, followed by additions of 750 μl of NTT (0.1 M NaCl, 50 mM Tris [pH 7.5], 2% Triton X-100) and 5 μl of antiserum to the *S. aureus* 60- and 70-kDa MBRP proteins. After shaking for 30 min, 250 μl of 10% protein A-Sepharose (Pharmacia) was added and the sample was slowly shaken for 6 h more at room temperature. Protein A-Sepharose was washed twice with NTT and once with 50 mM Tris (pH 6.8) and finally suspended in Laemmli sample buffer. Half of the sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (38). The 10% gel was dried and analyzed by autoradiography. ^{14}C-labeled methylated proteins (Amersham) were used as standards.

Western blotting. *E. coli* TG1 cells (5×10^7) were infected with the lambda gt10 clones sc2 and sc4 and with an intact lambda gt10 control, using a multiplicity of infection of 2. Cells were grown in L broth supplemented with 5 mM $CaCl_2$ and allowed to lyse. The lysate was stored at −20°C as aliquots. For Western blot (immunoblot) analysis, proteins were precipitated with 10% trichloroacetic acid, washed with acetone, and dissolved in Laemmli sample buffer. TG1 cells with and without pKTH1878, and *B. subtilis* BRB1 cells, were grown in L broth. Cell samples were taken at an A_{660} of 1.0. Cells were treated with 500 μg of lysozyme per ml for 5 min at room temperature, and Laemmli sample buffer was added. Proteins were separated by SDS-PAGE (10%), and the gel was immunoblotted (68) with each antisubunit antiserum obtained for the *S. aureus* MBRP and *B. subtilis* S complexes. Filters were stained with anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Protoblot; Promega).

DNA sequencing and sequence analysis. For DNA sequencing, plasmids were produced by a large-scale alkaline method (6) and purified by CsCl gradient centrifugation, followed by DNA sample preparation as described previously (27). Recombinant M13 phage DNAs were isolated as described earlier (45). DNA sequencing was carried out by the dideoxy-chain termination method (56), using [^{35}S]dATP (>1,000 Ci/mmol; Amersham) as the labeled nucleotide. The DNA polymerase used was Sequenase (U.S. Biochemicals),

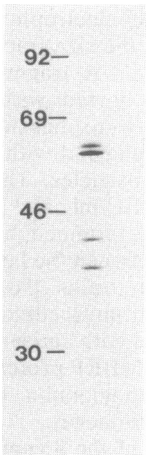


FIG. 1. Immunoprecipitation of the *B. subtilis* BRB34 cellular proteins. Cells were labeled with [^{35}S]methionine and immunoprecipitated with antiserum raised against the 60- and 70-kDa proteins of the *S. aureus* MBRP complex. Immunoprecipitate was analyzed by SDS-PAGE followed by autoradiography.

and the protocol recommended by the manufacturer was followed. From the pUC9 and M13mp18 subclones of the S complex, sequencing was done by the primer walking strategy. Sequence analysis was performed with the PCGENE set of programs (Genofit). SWISSPROT and EMBL were used as protein and nucleic acid sequence data banks, respectively. The protein sequence data bank was screened with the FASTP program (40), and protein sequence alignments were made by using the algorithm of Myers and Miller (46).

Mapping of the S-complex locus. To determine the chromosomal map position of the S-complex operon in *B. subtilis*, the integration vector pJH101 (19), PBS1 transduction, and the kit 4 mapping strain (QB943) were utilized as follows. The *Sph*I fragment of 740 base pairs (bp), located downstream from the S-complex operon (see Fig. 2), was cloned into pJH101, resulting in plasmid pKTH1916. Competent *B. subtilis* BRB1 cells were transformed with pKTH1916 DNA and selected for chloramphenicol resistance. Since pJH101 is unable to replicate in *B. subtilis*, pKTH1916 was forced to integrate to the chromosomal locus adjacent to the S-complex operon by homologous recombination. Southern blot analysis of the chromosomal integrants verified the integration event expected (data not shown). One of the clones was designated BRB693 and was used as the donor for PBS1 transduction. The PBS1 phage lysate, obtained after infection of the BRB693 donor, was used to infect the *B. subtilis* QB943 recipient cells. The transductants were selected for chloramphenicol resistance on L-agar–chloramphenicol plates and for *pyrD* or *trpC2* markers on minimal plates supplemented with appropriate amino acids and bases. Since the *B. subtilis* PDH genes *aceA* and *citL*, encoding the E1 and E3 subunits, are located at map positions 126 and 124° (72), respectively, only the kit 4 reference strain, carrying the selectable *pyrD* and *trpC2* markers at positions 135 and 205°, respectively, was used for mapping.

RESULTS

Cloning the *B. subtilis* S-complex operon. Antiserum raised against the 60-kDa protein of the *S. aureus* MBRP complex has been found to immunoprecipitate four proteins in *B. subtilis* (1). These cross-reacting *B. subtilis* proteins have been assumed to correspond to the *B. subtilis* S complex. In this study, we used antiserum raised against the *S. aureus* 60- and 70-kDa proteins, and also this antiserum immunoprecipitated the four proteins from *B. subtilis* cell lysate (Fig. 1). The molecular sizes of these *B. subtilis* proteins were 41, 37, 61, and 59 kDa (Fig. 1), being essentially the same as those reported earlier for the *B. subtilis* S-complex proteins (41, 36, 64, and 60 kDa [15]).

To clone the S-complex operon, a *B. subtilis* gene library was constructed with lambda phage gt10. The average size of DNA fragments inserted was 7 kb. After infecting the *E. coli* NM514 host, approximately 2,000 plaques from the library were screened for the S-complex proteins by immunoblotting with the above-mentioned rabbit antiserum to the *S. aureus* 60- and 70-kDa proteins. Immunoblotting of the plaques revealed five positive clones. All of these clones contained a 1.2-kb *Eco*RI fragment and were thus preliminarily considered to be overlapping. Two of the clones, denoted sc2 and sc4, were expected to cover the region containing the entire S-complex operon (Fig. 2). The inserts of the sc2 and sc4 clones were later subcloned as *Eco*RI fragments into pUC9 for further studies.

Southern blot and Western blot analyses of the S-complex operon. To confirm the *B. subtilis* chromosomal origin and colinearity of the inserts in lambda clones sc2 and sc4, Southern blot analysis was performed. Chromosomal DNA of *B. subtilis* BRB1 was digested with *Eco*RI, separated in an agarose gel, transferred to a nylon membrane, and hybrid-

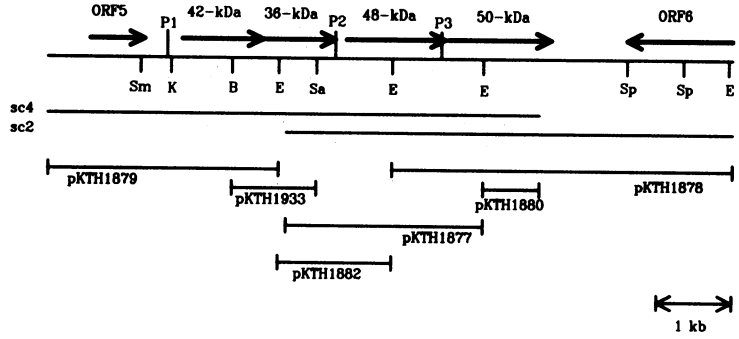


FIG. 2. Physical map and subclones of the S-complex operon from *B. subtilis*. Sc2 and sc4 refer to two overlapping lambda gt10 clones carrying the S-complex genes and pKTH numbers indicate the pUC9 subclones of the operon. Restriction enzyme sites for *Bam*HI (B), *Eco*RI (E), *Kpn*I (K), *Sal*I (Sa), *Sma*I (Sm), and *Sph*I (Sp) are marked. P1, P2, and P3 refer to promoters of the S-complex operon. The 42-, 36-, 48-, and 50-kDa proteins encoded by the operon are marked. The two adjacent ORFs are designated ORF5 and ORF6.

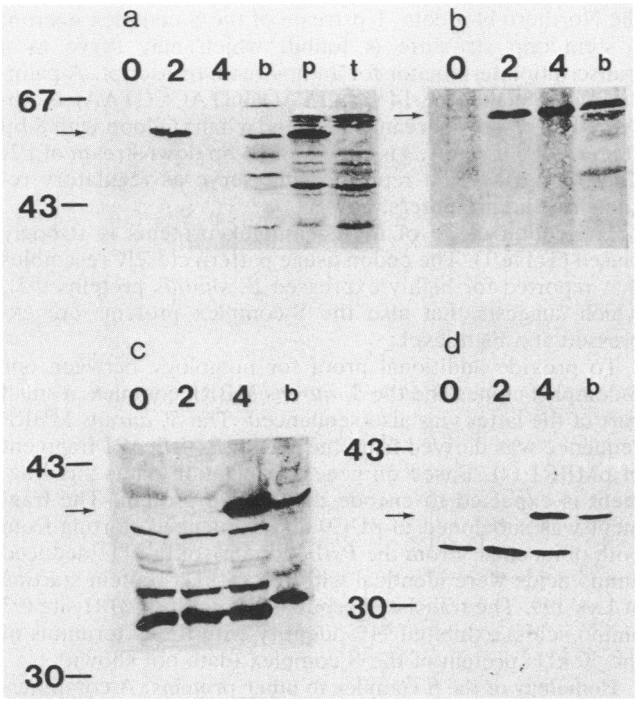


FIG. 3. Western blot of the S-complex clones, using antisera directed against the four individual *S. aureus* MBRP proteins. Filters a, b, c, and d were immunoblotted with antiserum against 60-, 70-, 46-, and 41-kDa MBRP complex proteins, respectively. Antisera were used at a dilution of 1:1,000. Lanes: 0, lambda gt10 control; 2, clone sc2; 4, clone sc4; b, *B. subtilis* BRB1; p, subclone pKTH1878 in *E. coli* TG1; t, TG1 control cells. As samples, 250 μl of lambda lysate, 25 μl of BRB1 cells, and 10 μl of *E. coli* cells were used, except for gel c, when 1 ml of lysate and 75 μl of BRB1 cells were added. The S-complex proteins are indicated by arrows.

ized with the nick-translated sc2 and sc4 DNA. The approximate sizes of the chromosomal *Eco*RI fragments, identified by the sc2 and sc4 probes, were 1.2, 1.6, and 3.2 kb and 1.2, 1.7, and 6.5 kb, respectively (data not shown). The 3-kb insert of the pKTH1879 subclone of sc4 obviously forms part of the 6.5-kb chromosomal fragment, found by the sc4

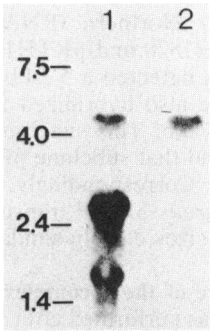


FIG. 5. Northern blot of the *B. subtilis* S complex. Total RNA from *B. subtilis* BRB1 was analyzed by nick-translated pKTH1878 (lane 1) and pKTH1879 (lane 2) probes.

probe. Thus, the organization of the *Eco*RI fragments of interest in the *B. subtilis* chromosomal DNA is similar to that of our lambda clones (Fig. 2). *Pst*I and *Cla*I digestions of the chromosomal DNA also revealed bands in Southern blots corresponding to the restriction sites found in the DNA sequence of the S complex (data not shown).

To determine whether the sc2 and sc4 clones expressed all of the S-complex proteins, *E. coli* TG1 cells were infected with them and analyzed by Western blotting with antisera separately raised against the four proteins of the *S. aureus* MBRP complex (Fig. 3). In the cells infected with sc4, the anti-46-kDa and anti-41-kDa antisera recognized a 43- and a 36-kDa protein, respectively. Anti-70-kDa antiserum detected a 64-kDa protein in the cells infected either with sc4 or sc2, whereas anti-60-kDa antiserum recognized a 60-kDa protein only in the cells infected with sc2 or carrying an sc2 subclone, pKTH1878. Proteins of the same size as those of the lambda clones were also detected from *B. subtilis* cell lysate.

The four antisera against the individual proteins of the *B. subtilis* S complex also recognized the same four proteins, thus confirming the S-complex origin of our clones (Fig. 4). This is the first immunological data showing the homology between the individual proteins of these two complexes.

Northern blot analysis of the S complex. To analyze the transcripts of the S complex, total RNA of *B. subtilis* BRB1

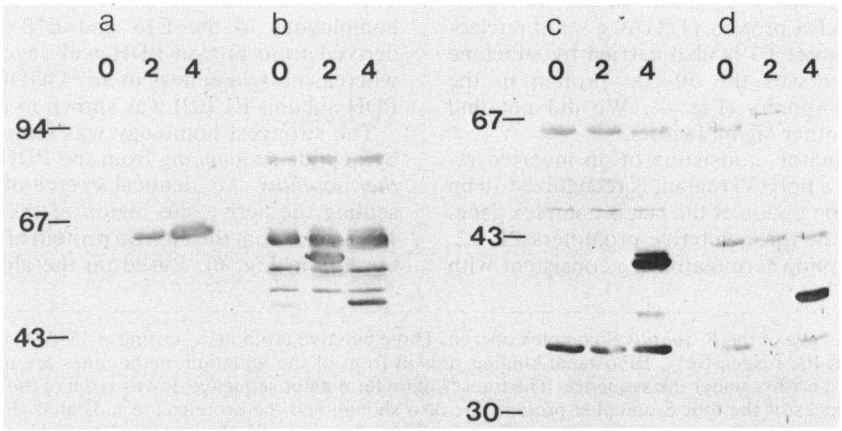


FIG. 4. Western blot of the S-complex lambda clones with antisera separately raised against the four individual *B. subtilis* S-complex proteins. Filters a, b, c, and d were immunoblotted with antiserum to 64-, 60-, 41-, and 36-kDa S-complex proteins, respectively. For lanes, see legend to Fig. 3. Purified immunoglobulin fraction was used at a concentration of 4 μg/ml. For each lane, 250 μl of lambda lysate was used as the sample.

was hybridized after Northern (RNA) blotting with the nick-translated pKTH1878 and pKTH1879 probes (Fig. 5). Both of these probes detected a 5.2-kb transcript, whereas the pKTH1878 probe also hybridized to two smaller transcripts of 2.8 and 1.7 kb. This suggests that there are two internal promoters and that subclone pKTH1878 is at the 3′ end of the operon. Correspondingly, the Northern blot analysis of the *S. aureus* MBRP transcripts revealed three mRNA species with sizes closely similar to those observed here (4).

Nucleotide sequence of the S complex. Sequencing of the sc2 and sc4 inserts was performed after subcloning in pUC9. Both strands of the plasmids were sequenced by the primer walking method. One of the strands was also sequenced from M13mp18 subclones, except for the region included in pKTH1879, which contains the promoter of the S complex. The combined sequence is shown in Fig. 6. The sequence of the S-complex operon contains four open reading frames coding for proteins of 41.6, 35.5, 47.5, and 49.7 kDa (Fig. 2 and 6), herein designated 42-, 36-, 48-, and 50-kDa proteins, respectively. The genomic order of the S-complex proteins is the same as that of the homologous *S. aureus* proteins (4). The sizes of the two smallest proteins of the S complex, as deduced from the sequence, are with reasonable accuracy the same as those observed by SDS-PAGE. In contrast, the 48- and 50-kDa proteins have an abnormally low mobility in the SDS-PAGE gel system, resulting in apparent molecular weights of 64,000 and 60,000, respectively (Fig. 3 and 4).

The first three of the S-complex proteins are translated from the same frame. The intergenic distances between the adjacent genes encoding the 42-, 36-, 48-, and 50-kDa proteins are 3, 114, and 4 bp, respectively. Upstream of each of the four genes there is a ribosome-binding site complementary to the 3′-end sequence of 16S rRNA (24).

Approximately 80 bp upstream of the ribosome-binding site of the 42-kDa protein, a putative promoter sequence, P1 (TTGgCA < 19 nucleotides > TATAAT), can be found (Fig. 6). Except for a single nucleotide, P1 is identical to the consensus sequence of the *B. subtilis* sigma-43 promoter (18). The putative promoter P2 can be recognized ca. 100 bp upstream of the ribosome-binding site for the 48-kDa protein (TTGAaA < 21 nucleotides > TtTAAT). In this case, there is a difference in two nucleotides from the consensus sequence and, furthermore, the distance between the −35 and −10 regions is unusually long. The third putative promoter, P3, can be observed ca. 40 bp upstream of the ribosome-binding site for the 50-kDa protein (TcGACg < 20 nucleotides > TtaAAT). Promoter P3 is also carried by subclone pKTH1878, which expresses the 50-kDa protein in the absence of external promoters (Fig. 2). We did not find putative promoters for other sigma factors.

A transcription terminator, consisting of an inverted repeat (14 bp) followed by a poly(T) region, is recognized 10 bp downstream from the stop codon of the last S-complex gene. The distances between the three putative promoters P1, P2, and P3 and the transcription terminator are consistent with the Northern blot data. Upstream of the S-complex operon, a stem-loop structure is found, which may serve as a transcription terminator for an upstream transcript. A palindromic sequence of 14 bp (TTAGGGTACCCTAA) is observed 20 bp downstream of P1, and a hairpin loop with 8-bp stem (AGAGGGAA..) is observed 16 bp downstream of P2. These long inverted repeats might serve as regulatory regions for the promoters.

The codon usage of the S-complex proteins is strongly biased (Table 1). The codon usage pattern closely resembles that reported for highly expressed *B. subtilis* proteins (57), which suggests that also the S-complex proteins are expressed at a high level.

To provide additional proof for homology between our S-complex clones and the *S. aureus* MBRP complex, a small part of the latter was also sequenced. The *S. aureus* MBRP sequence was derived from the 1.1-kb *Pst*I-*Eco*RI fragment of pMBR1 (4). Based on gene organization data, this fragment is expected to encode the 71-kDa protein. The fragment was subcloned to pUC9 and sequenced starting from both of its ends. From the *Pst*I end, 56% of the 111 deduced amino acids were identical with the 48-kDa protein starting at Lys-199. The translated sequence from the *Eco*RI site (93 amino acids) exhibited 71% identity with the N terminus of the 50-kDa protein of the S complex (data not shown).

Homology of the S complex to other proteins. A computer-aided search for homologous proteins was performed. Table 2 shows that significant sequence homology (26 to 70%) was found between the *B. subtilis* S-complex proteins and the subunits of PDH, branched-chain 2-oxoacid dehydrogenase (BCDH), and 2-oxoglutarate dehydrogenase (OGDH) multiprotein complexes of different origins. PDH, BCDH, and OGDH consist of subunits termed E1 (or E1α and E1β), E2, and E3. The E3 subunit is common for all PDH, OGDH, and BCDH complexes identified, except for *Pseudomonas putida*, in which three different E3 proteins have been found (12). The E2 subunits are complex specific but still partly homologous to each other. The E1 subunits react with the primary substrate of the complex and are therefore most diverged in these complexes. All four S-complex proteins shared homology with the subunits of the human PDH and *P. putida* BCDH. Furthermore, the 50-kDa S-complex protein was found to be strongly homologous to the E3 subunits and the 48-kDa protein was strongly homologous to the E2 subunits of several PDH, BCDH, and OGDH complexes. The 42- and 36-kDa proteins were found to be closely homologous to the E1α and E1β subunits, respectively, derived from human PDH and several BCDH complexes, whereas no relatedness to any OGDH subunits or to *E. coli* PDH subunit E1 (62) was shown in these proteins.

The strongest homology was found to sequences derived by peptide sequencing from the PDH subunits of *B. stearothermophilus*. An identical stretch of 14 amino acids, representing the active-site region of the PDH subunit E3 (51), was found from the 50-kDa protein of the S complex (marked by dots in Fig. 6). Based on the alignment, the active-site

FIG. 6. Nucleotide sequence of the *B. subtilis* S-complex operon. Three putative promoters starting at 1548, 3742, and 5134 are underlined and marked as P1, P2, and P3, respectively. Ribosomal binding sites in front of the initiation methionines are underlined. Major inverted repeats are shown by dotted arrows under the sequence. The transcription terminator sequence downstream of the operon starts at 6644. The deduced amino acid sequences of the four S-complex proteins are also shown, and the proteins are indicated above the sequence by their molecular sizes (42, 36, 48, and 50 kDa). E1α, E1β, E2, and E3 refer to the *B. subtilis* PDH subunits identified by homology. A stretch of 14 amino acids, identical with the active-site region of PDH subunit E3 of *B. stearothermophilus* (51), is marked by a dotted line starting at Leu-43 in the 50-kDa protein. The putative lipoyl binding residue, Lys-43, in the 48-kDa protein is marked by an asterisk. Numbering refers to the *B. subtilis* sequence in the sc2 and sc4 clones carrying also the flanking regions of the S-complex operon. The entire sequence has been submitted to the EMBL/Genbank nucleotide sequence data base (accession number M31542).

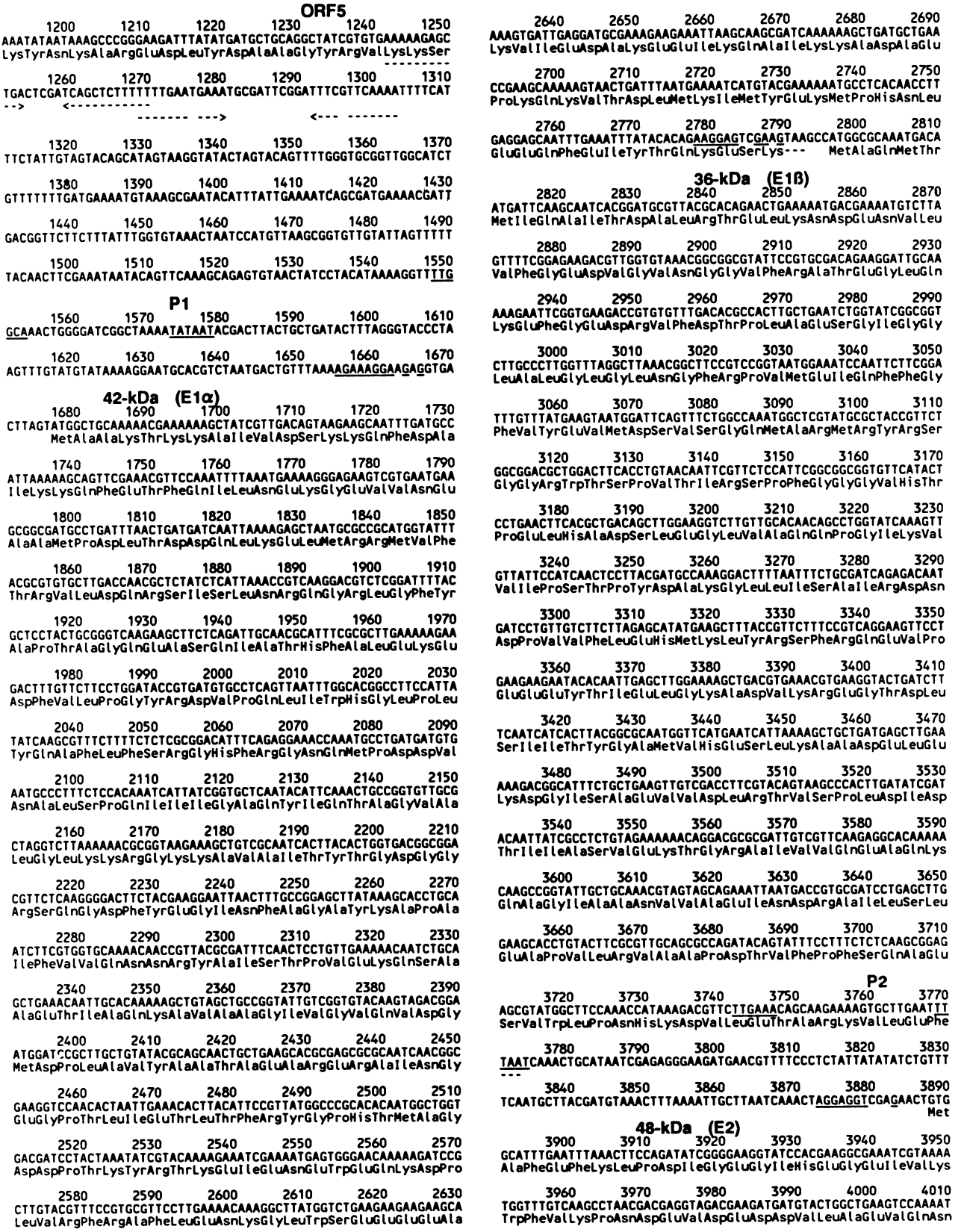
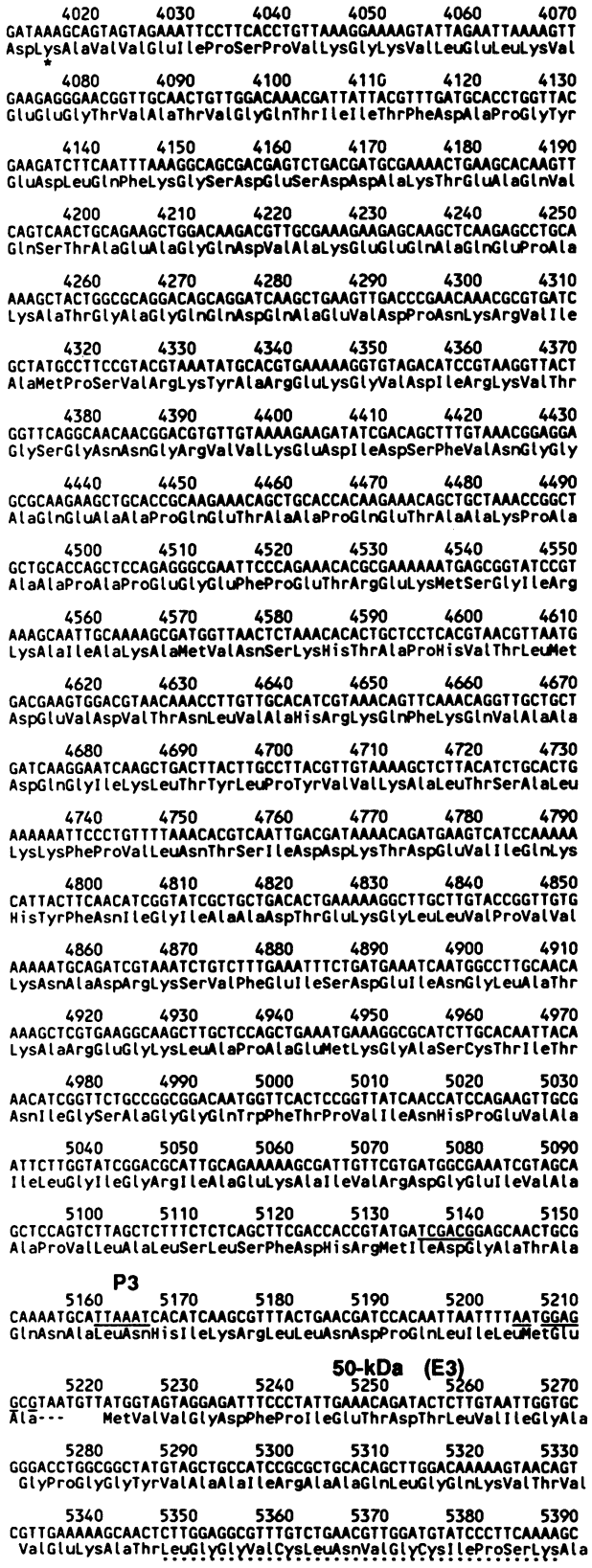


FIG. 6—*Continued*



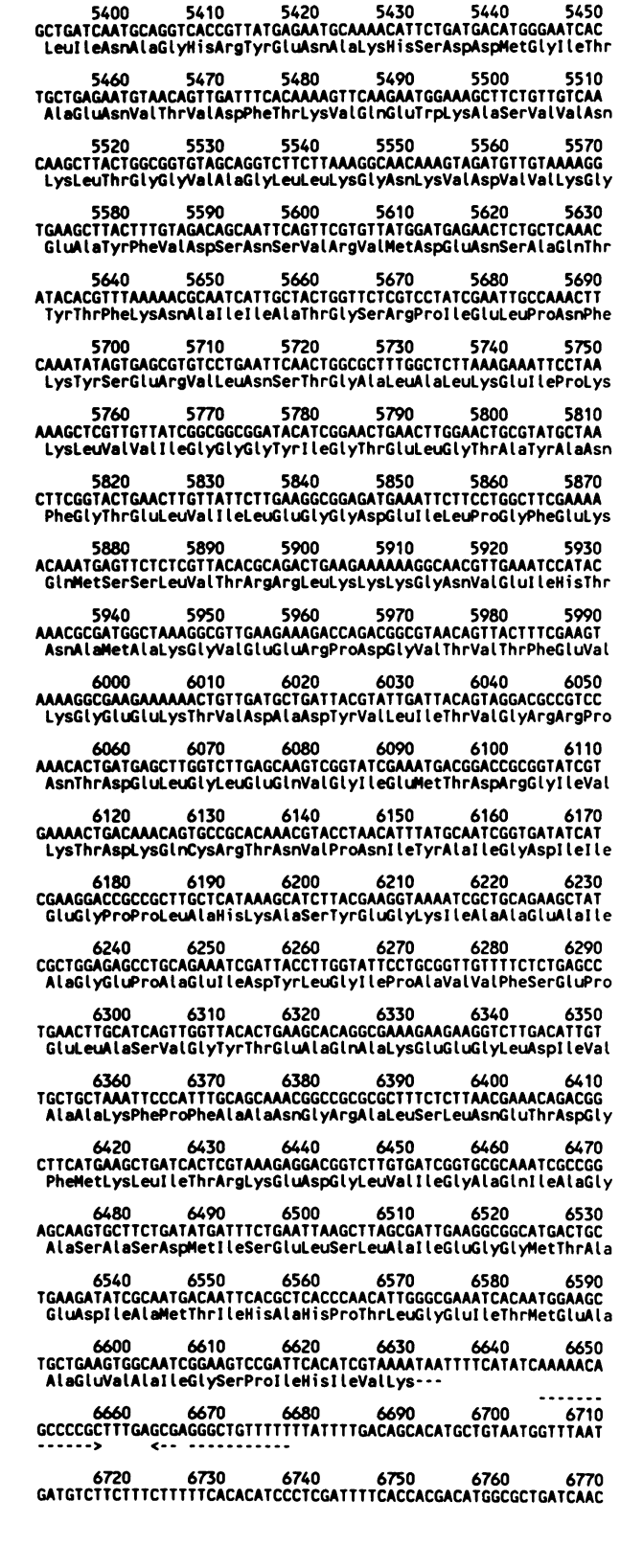


FIG. 6—*Continued*

disulfide bridge in the 50-kDa protein is Cys-47–Cys-52. The N-terminal 211-amino-acid fragment available from the E2 subunit (50) was closely related to the N terminus of the 48-kDa protein of the S complex (Table 2). When the sequences corresponding to the functional regions of the E2 are compared, an even higher degree of homology is found. In the lipoyl domain (85 amino acids) and in the E1/E3 binding domain (43 amino acids), 87 and 81% amino acid identity is shared, respectively. Lys-43, corresponding to the lipoyl binding residue of the E2 protein, is marked in the sequence by an asterisk (Fig. 6). Recently, a part of the E1α subunit of the *B. stearothermophilus* PDH (48 amino acids), corresponding to the thiamine pyrophosphate-binding region has been published (28). Except for three amino acids, this sequence is identical to the 42-kDa protein starting at Gly-165.

Based on the homology data, we conclude that the S complex may be identified as the *B. subtilis* PDH. The *B. subtilis* PDH has been previously isolated and shown to contain four subunits with very similar sizes to those of the S complex (32). The *B. subtilis* PDH complex also possesses BCDH activity (41).

Biochemical identification of PDH. Our attempts to construct a clone carrying the whole S-complex operon have been unsuccessful so far. This may be due to harmful effects caused by the S-complex proteins in a multicopy vector system. For example, the plaques of the lambda clone sc4 were significantly smaller than those of clone sc2 or the intact lambda gt10. Thus, we were unable to determine the enzymatic activity of the entire cloned operon.

We have also analyzed the sc2 and sc4 lambda clones by Western blotting with antisera raised against PDH of *B. subtilis* and *B. stearothermophilus*. Both of these antisera detected the lambda clones as positive (data not shown).

Mapping of the S-complex operon. The *B. subtilis* genes *aceA* and *citL*, corresponding to PDH subunits E1 and E3, have been separately mapped to chromosomal positions 126° (7, 72) and 124° (31, 72) respectively. In this study, we mapped by PBS1 transduction the *cat* gene inserted immediately downstream from the S-complex operon. A linkage of 43% to the *pyrD* locus (135°) of the recipient strain was obtained, indicating a similar position of the S-complex operon to that of PDH. No linkage to the control locus (*trpC*, 205°) was observed. Thus, the chromosomal map position of the S complex is consistent with the identity to the PDH operon. Insertional inactivation of the operon with a 740-bp fragment of the E2 gene, starting at 3,990 bp, was unsuccessful, which suggests that inactivation of the operon is lethal to *B. subtilis* BRB1 cells (data not shown).

ORFs of the flanking regions. In addition to the S-complex genes, the cloned sequence revealed two other open reading frames (ORFs) located upstream (ORF5; 26 kDa) and downstream (ORF6) from the S-complex operon (Fig. 2). The amino acid sequence deduced from ORF6 contains two strongly hydrophobic regions, suggesting that it is part of a transmembrane protein. ORF5 and ORF6 were not found to have any major homology to sequences presented in the protein and DNA sequence data banks.

DISCUSSION

In this work, we have cloned into lambda gt10 the operon encoding the *B. subtilis* S complex. The *B. subtilis* gene library was screened with antiserum prepared against the 60- and 70-kDa proteins of the homologous *S. aureus* MBRP complex. Two of the positive clones, sc2 and sc4, were

TABLE 1. Codon usage of the S-complex proteins compared with *B. subtilis* proteins with high or average expression level

Codon[a]		No. used					
		S-complex protein				High[b]	Avg[c]
		42 kDa	36 kDa	48 kDa	50 kDa		
Ala	GCT	15	8	24	23	40	176
	GCC	5	4	1	2	1	129
	GCA	12	9	23	17	11	201
	GCG	10	8	8	9	5	185
Arg	CGT	10	11	11	6	23	105
	CGC	7	5	3	6	14	85
	CGA*	0	0	0	0	0	41
	CGG	0	0	0	0	1	59
	AGA	1	2	0	2	10	125
	AGG*	0	0	0	0	0	37
Asn	AAT	4	4	5	5	10	204
	AAC	8	4	12	14	24	179
Asp	GAT	13	9	16	14	5	312
	GAC	7	11	14	8	12	191
Cys	TGT*	0	0	0	2	0	30
	TGC*	0	0	1	1	0	34
Gln	CAA	20	10	16	6	28	183
	CAG	4	2	5	3	6	174
Glu	GAA	26	24	32	33	17	536
	GAG	7	5	7	9	7	254
Gly	GGT	10	10	8	17	21	157
	GGC	5	11	11	19	17	213
	GGA	10	7	12	15	22	193
	GGG	1	0	1	0	1	87
His	CAT	2	4	3	3	6	132
	CAC*	3	1	5	4	0	80
Ile	ATT	14	10	11	14	7	327
	ATC	10	10	18	24	14	265
	ATA	0	0	0	0	1	73
Leu	TTA	8	7	9	2	14	183
	TTG*	0	3	2	4	0	114
	CTT	12	16	9	20	12	220
	CTC	1	0	1	2	2	94
	CTA	3	0	0	0	7	50
	CTG	0	2	4	5	1	207
Lys	AAA	23	12	32	27	31	461
	AAG	9	1	5	4	3	166
Met	ATG	10	9	7	11	9	216
Phe	TTT	7	4	7	3	8	227
	TTC	10	10	6	9	14	118
Pro	CCT	9	8	9	10	7	102
	CCC*	0	0	0	0	0	31
	CCA	3	6	9	5	5	57
	CCG	4	1	5	3	1	144
Ser	TCT	7	9	8	10	24	128
	TCC*	0	0	1	0	0	78
	TCA	2	5	4	4	11	124
	TCG*	1	0	0	0	0	58
	AGT	1	0	0	4	3	59
	AGC	0	4	4	2	5	140
Thr	ACT	9	5	10	16	15	80
	ACC*	0	0	0	0	0	83
	ACA	7	10	9	14	13	212
	ACG	4	2	5	2	3	146
Trp	TGG	3	2	2	1	1	47
Tyr	TAT	5	1	1	5	1	181
	TAC	8	5	4	7	2	113
Val	GTT	4	15	18	21	16	182
	GCT	3	4	5	4	3	171
	GTA	7	12	15	16	12	144
	GTG	7	3	4	3	7	145

[a] Codons not used in highly expressed proteins are marked by asterisks.
[b] High represents codons for proteins that are expressed at high level, such as ribosomal proteins (HI BIAS in reference 57).
[c] Avg represents codons for average proteins (OTHERS in reference 57).

TABLE 2. Amino acid homology between the *B. subtilis* S-complex proteins and closely related proteins

S-complex protein (kDa)	Identical amino acids (%)	Homologous protein	Reference(s)
42	26	E1α, PDH, human	17, 37
	30	E1α, BCDH, *P. putida*	9
	33	E1α, BCDH, rat	73
	31	E1α, BCDH, human	20
	31	E1α, BCDH, bovine	35
36	36	E1β, PDH, human	37
	47	E1β, BCDH, *P. putida*	9
48	32	E2, PDH, human	67
	36	E2, BCDH, *P. putida*	10
	70	E2, PDH, *B. stearothermophilus*[a]	50
	35	E2, PDH, *A. vinelandii*	26
	34	E2, PDH, *E. coli*	63
	27	E2, PDH, *S. cerevisiae*	47
	30	E2, BCDH, bovine	22, 39
	26	E2, BCDH, human	39
	32	E2, OGDH, *E. coli*	60
	36	E2, OGDH, *B. subtilis*	13
50	42	E3, human	49, 54
	42	E3-val, *P. putida*	11
	46	E3, *E. coli*	64
	43	E3, pig	49
	39	E3, *S. cerevisiae*	8

[a] A fragment of 211 amino acids.

found to be overlapping. In Western blotting, using antisera to the *S. aureus* MBRP complex proteins, the two clones were found to express four proteins of 43, 36, 64, and 60 kDa. These proteins were also detected with the same antisera from the cell lysate of *B. subtilis*. Moreover, antisera raised against the four *B. subtilis* S-complex proteins detected the same four proteins from cells infected with sc2 and sc4. Thus, the immunological data confirmed that the *B. subtilis* S complex and the *S. aureus* MBRP complex are homologous at the level of individual proteins and that our two lambda clones code for the *B. subtilis* S-complex genes.

The sequence analysis of the S-complex operon revealed four genes encoding proteins of 42, 36, 48, and 50 kDa. The order of the genes is the same as that of the homologous genes in the *S. aureus* MBRP operon, which encodes the 46-, 41-, 71-, and 60-kDa proteins (4). Promoters P1, P2, and P3, recognized from the sequence, were located in front of the entire operon, the third gene, and the last gene, respectively. A transcription terminator was found downstream from the last gene. In accordance with the sequence data, Northern blot analysis revealed transcripts of 5.2, 2.8, and 1.7 kb. This is consistent with the Northern blot data of the *S. aureus* MBRP similarly showing three transcripts of 5.9, 2.8, and 1.6 kb (4).

Comparison of the *B. subtilis* S-complex proteins with the sequences of the protein data bank revealed strong homology to the subunits of PDH, OGDH, and BCDH complexes of different origin. Most consistent homology was observed to the subunits of PDH and BCDH. Furthermore, the 211 amino acids derived by peptide sequencing from the E2 subunit of the *B. stearothermophilus* PDH had 70% identity with the N terminus of the 48-kDa S-complex protein. Thus, the homology data show that the S complex is obviously identical to the *B. subtilis* PDH complex, which also possesses BCDH activity (41). PDH connects the glycolysis to the tricarboxylic acid cycle, and BCDH catalyzes a step in the oxidation of branched-chain amino acids (53, 70). The *B. subtilis* PDH complex consists of subunits denoted E1α, E1β, E2, and E3 (32), corresponding to the 42-, 36-, 48-, and 50-kDa S-complex proteins, respectively. E1 is pyruvate decarboxylase (EC 1.2.4.1), E2 is dihydrolipoamide acetyltransferase (EC 2.3.1.12), and E3 is dihydrolipoamide dehydrogenase (EC 1.8.1.4; formerly EC 1.6.4.3).

B. subtilis OGDH and PDH utilize one common subunit, E3 (31), the gene of which has been localized close to the PDH E1 gene (72). This suggests that PDH subunit E1 is encoded by the same operon as E3. Consistently, the S-complex operon encodes the 50-kDa protein identified as E3 subunit and the 42- and 36-kDa proteins identified as E1α and E1β, respectively. Moreover, we found that a fragment adjacent to the S-complex operon mapped close to the chromosomal position of the genes encoding subunits E1 and E3 of PDH. Insertional inactivation of the E2 gene was not successful, indicating that the S-complex operon is essential. The same was also found with the insertional inactivation of the MBRP complex (4). However, there are *B. subtilis* strains with mutations in the E1 and E3 subunit genes. In an *aceA1* mutant, both E1α and E1β subunits are present but they have low binding affinity to the E2-E3 subcomplex (32). A *citL22* mutant has 6% of the E3 activity left but does not possess measurable PDH and OGDH activities (31). The viability of these mutants may be due to a small residual activity.

Some properties of the S complex are similar to those of the PDH, which has been purified from *B. subtilis* (32, 69) and from a closely related species, *B. stearothermophilus* (29, 30). The *B. subtilis* and *B. stearothermophilus* PDH is a complex consisting of four protein subunits. The molecular weights of the *B. subtilis* PDH subunits are 42,000, 38,000, 66,000, and 63,000 on a 12.5% acrylamide gel with Tris-glycine buffer and 42,000, 36,000, 59,000, and 54,000 on a 7.5% gel with phosphate buffer (32). Thus, the sizes are reasonably similar to those of the S-complex proteins. Very similar sizes have been found also for the *B. stearothermophilus* PDH subunits (29). The 48-kDa S-complex protein shows an abnormally low mobility in the SDS-PAGE gel system, which has been observed also for other E2 subunits of different origin. In the case of *E. coli*, the retarded mobility of the E2 subunit of PDH has been attributed to the swollen structure of the lipoyl binding domain or to the presence of proline- and alanine-rich regions in the protein, which cause decreased binding of SDS (53).

The *B. subtilis* PDH exists as a complex which has been reported to sediment at 73S (32). The *B. stearothermophilus* PDH complex sediments at 75S, and in electron microscopy these complexes have a diameter of 40 nm (29). Similarly, the S complex, released from the ribosomes by a low concentration of Mg^{2+} ions, can be recovered as particles sedimenting at 76S and having a diameter of 45 nm in electron microscopy (14). Furthermore, the PDH complex from *B. stearothermophilus* shows a striking resemblance to the mammalian (mitochondrial) PDH in terms of morphology, subunit composition, and molecular weight, sharply contrasting with the *E. coli* PDH, which consists of only three subunits (29). Consistently, we found a high level of homology between the 42- and 36-kDa S-complex proteins and the mammalian PDH subunits E1α and E1β, respectively, but not to the *E. coli* PDH subunit E1.

Why is PDH found to be attached to membrane-bound ribosomes as reported for the MBRP and S complexes?

Recently, much data have accumulated suggesting that the intracellular enzymes often exist as multienzyme complexes or are bound to the structural elements of the cell (61). For instance, mitochondrial PDH has a specific interaction with citrate synthase (65). Furthermore, mitochondrial PDH binds efficiently to the mitochondrial inner membrane, and this has been attributed to a specific interaction between PDH and complex I of the respiratory chain (66). Consistently, a large (60%) proportion of the *S. aureus* PDH (MBRP) is associated to the membranes at postexponential phase (2, 3), although previous data on the location of the PDH enzymatic activity are not available. Some reactions of energy production and consumption may occur in the same location of membrane, explaining the presence of PDH and ribosomes in the complexed fraction. However, the strong direct interaction between PDH and membrane-bound ribosomes, which has been observed in studies of the MBRP complex, is an interesting observation that is not easy to explain. The degree of this interaction is also correlated with the level of protein secretion. The increased binding of MBRP to the membrane fraction, concomitant with the increased secretion during the postexponential growth phase, has been considered to indicate direct participation of MBRP in protein secretion (3). However, the phenomenon may be unrelated to the secretion event and instead may be due to changes in energy metabolism of cells entering the stationary growth phase.

PDH and OGDH are closely related enzymes, and *B. subtilis* OGDH and PDH share one subunit, E3 (31). That E3 is common to both PDH and OGDH gives a rationale to the third promoter (P3) of the PDH operon, identified in this study. Both PDH and OGDH are regulated by substrate induction (21, 48), and promoter P3 may thus make it possible to express the last gene independently from the other genes of the PDH operon. The reason for having still one promoter (P2) in front of the gene encoding the 48-kDa protein (E2) is not as obvious.

Based on the data presented here, we conclude that the *B. subtilis* S complex is identical to the PDH complex, an enzyme in intermediary metabolism. It seems unlikely that PDH would also have an additional function as a primary component in protein secretion. However, an indirect role of PDH in protein secretion is highly probable due to the crucial function of the enzyme. In conclusion, we propose that the S complex does not have a direct role in the protein secretion machinery, in contrast to what was expected in the beginning of this study.

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