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Characterization of a Chimeric *proU* Operon in a Subtilin-Producing Mutant of *Bacillus subtilis* 168

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The ability to respond to osmotic stress by osmoregulation is common to virtually all living cells. Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium* can achieve osmotolerance by import of osmoprotectants such as proline and glycine betaine by an import system encoded in an operon called *proU* with genes for proteins ProV, ProW, and ProX. In this report, we describe the discovery of a *proU*-type locus in the gram-positive bacterium *Bacillus subtilis*. It contains four open reading frames (ProV, ProW, ProX, and ProZ) with homology to the gram-negative ProU proteins, with the *B. subtilis* ProV, ProW, and ProX proteins having sequence homologies of 35, 29, and 17%, respectively, to the *E. coli* proteins. The *B. subtilis* ProZ protein is similar to the ProW protein but is smaller and, accordingly, may fulfill a novel role in osmoprotection. The *B. subtilis proU* locus was discovered while exploring the chromosomal sequence upstream from the *spa* operon in *B. subtilis* LH45, which is a subtilin-producing mutant of *B. subtilis* 168. *B. subtilis* LH45 had been previously constructed by transformation of strain 168 with linear DNA from *B. subtilis* ATCC 6633 (W. Liu and J. N. Hansen, J. Bacteriol. 173:7387–7390, 1991). Hybridization experiments showed that LH45 resulted from recombination in a region of homology in the *proV* gene, so that the *proU* locus in LH45 is a chimera between strains 168 and 6633. Despite being a chimera, this *proU* locus was fully functional in its ability to confer osmotolerance when glycine betaine was available in the medium. Conversely, a mutant (LH45 Δ*proU*) in which most of the *proU* locus had been deleted grew poorly at high osmolarity in the presence of glycine betaine. We conclude that the *proU*-like locus in *B. subtilis* LH45 is a gram-positive counterpart of the *proU* locus in gram-negative bacteria and probably evolved prior to the evolutionary split of prokaryotes into gram-positive and gram-negative forms.

Bacteria are capable of active osmoregulatory responses which allow them to adapt to large fluctuations in the osmolarity of their environment (5, 12, 37, 39, 46). Study of osmoregulation has important applications to food microbiology (42, 43), plant-microbe interactions (7, 21), and medical microbiology (3, 4, 8, 18, 19, 23). The ability of cells to provide gene regulatory responses to changes in a physicochemical parameter rather than a specific molecule requires a novel signal transduction mechanism (11). These responses to osmotic stress are often crucial to survival, and the mechanisms by which this is achieved have been studied extensively, particularly in the gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium* (2, 6, 13–16, 20, 22, 24). A central response to osmotic stress is the uptake of molecular species that function as osmoprotectants, such as proline and *N*,*N*,*N*-trimethylglycine (glycine betaine). Although several osmoprotectant uptake systems have been identified, one of the most extensively studied is the osmoregulatory locus known as *proU*, which is an operon that encodes a high-affinity transport system, consisting of three proteins (ProV, ProW, and ProX) with glycine betaine as a major substrate, that is found both in *E. coli* (14, 15, 22, 25, 26, 34, 37–39) and *S. typhimurium* (6, 29, 40, 45). ProV and ProW are membrane-associated proteins, and ProV shares considerable sequence identity with ATP-binding proteins from other periplasmic systems. ProX encodes the periplasmic glycine betaine-binding protein (25, 38, 45). Glycine betaine also functions as an osmoprotectant in gram-positive bacteria (5, 27), and although it has been demonstrated that there are at least two systems for glycine betaine uptake in *Bacillus subtilis* (5), the genes responsible for osmoregulation in gram-positive bacteria have not been identified; this has been a barrier to genetic studies of their osmoregulation.

In this report, we describe the discovery of a *proU*-like locus in *B. subtilis*. The discovery is serendipitous in that it occurred in the course of studying gene sequences that lie upstream from the *spa* operon, which contains genes for the biosynthesis of the antimicrobial peptide subtilin. To facilitate studies of the genes involved in subtilin biosynthesis, workers in this laboratory had previously converted *B. subtilis* 168 from the wild-type nonproducing strain to a subtilin-producing mutant, LH45 (35). This was achieved by transforming strain 168 with linear DNA from subtilin-producing *B. subtilis* ATCC 6633 and forcing a double recombination between the linear DNA and the strain 168 chromosome because of a selectable marker (the *cat* gene) that was present in the *spa* operon of strain 6633 (35). LH45 was found to be a subtilin-producing mutant, and further characterization established that the *spa* operon had been integrated into the chromosome (35). Since the *spa* genes are unique to strain 6633, their integration into the strain 168 chromosome must have involved homologies that flanked both sides of the *spa* operon, suggesting that the *spa* operon in strain 6633 is flanked by genes that are also present in strain 168; it is these genes that provided the homologies required for the double recombination to occur (35). This work is the result of our attempt to define the region of homology in which the recombination occurred. When we sequenced this region of homology, we found that it was within a gene that encodes a protein that is very similar to the ProV protein in the *E. coli proU* locus. We also found homologs to the ProW and ProX proteins, in addition to a novel gene which we have named *proZ*. Because the site of recombination is in the *proV* gene,

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the *proU*-like locus in strain LH45 is actually a chimera in which the upstream portion of the *proV* gene is derived from strain 168, with the rest of the *proU* locus being derived from strain 6633. We performed experiments that show that this chimeric *proU*-like locus confers osmotolerance in the presence of glycine betaine, as would be expected if it encoded a glycine betaine import system. We conclude that this is a functional *proU* locus, and its availability permits genetic studies of osmoregulation in a gram-positive bacterium.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM101 and JM109 were used for subcloning and preparation of single-stranded DNA. *B. subtilis* ATCC 6633 was obtained from the American Type Culture Collection (Rockville, Md.). *B. subtilis* LH45 and LH45Δc are previously constructed derivatives of *B. subtilis* 168 (35). The vectors used in this study were pTZ18, pTZ19, and M13 phage-derived mp18 and mp19.

Media and growth conditions. *B. subtilis* strains used throughout this study were maintained on Difco Penassay broth (PAB) agar plates. Studies of osmolarity effects on growth were performed in a defined medium that contained $(NH_4)_2SO_4$ (2 g/liter), K_2HPO_4 (14 g/liter), KH_2PO_4 (6 g/liter), Na_3-citrate-$2H_2O$ (1 g/liter), $MgSO_4$-$7H_2O$ (0.2 g/liter), glucose (5 g/liter), and the supplemental amino acids phenylalanine (20 mg/liter), tryptophan (20 mg/liter), and methionine (20 mg/liter). In some experiments, 0.1% PAB (which is a source of osmoprotectants) was employed instead of the glucose and supplemental amino acids. The osmotic strength of the medium was adjusted by addition of NaCl from a 5 M stock solution. Media of different osmolarities were inoculated with a 2% volume of an overnight culture of *B. subtilis* grown in medium that was the same as that used for the growth experiment (except that NaCl was omitted). When glycine betaine (Sigma, St. Louis, Mo.) was employed, its concentration was 1 mM. The cultures were incubated in a rotary shaker at 200 rpm and 37°C. Growth was monitored with a Klett-Summerson colorimeter, and the morphology of the cells was determined by phase-contrast microscopy.

Construction of a mutant with a deletion within the *proU* region of LH45. The *spa* operon with its upstream flanking sequence was previously cloned as a 9.6-kb *Pst*I-*Pst*I fragment in plasmid pTZ19U (9). A 5.3-kb *Eco*RV-*Eco*RV fragment (see Fig. 1) was subcloned into the blunt *Hin*cII site of pTZ19U (destroying the *Hin*cII site) to generate plasmid pEV. Next, a 3.1-kb segment containing nearly all of the *proU* operon was removed by digesting pEV with *Hin*cII and then religating it to generate plasmid pEVΔH_2, from which the *Hin*cII fragment had been deleted. A *cat* gene (36) was cloned into the *Hin*cII site of pEVΔH_2. This plasmid was linearized by restriction with *Bam*HI and *Pst*I, introduced into *cat*-free LH45 strain LH45ΔC (36) by transformation, and selected on chloramphenicol plates. Colonies are the result of transformants in which the *cat* gene has been incorporated into the chromosome by a double recombination between flanking homologies, which has the effect of deleting the *proU* locus and replacing it with a *cat* gene. The chromosomal DNA of recombinants was examined by Southern blot analysis to confirm that the recombination occurred as expected. One of these recombinants, designated mutant LH45Δ*proU*, was used to characterize the effects of a deletion in the *proU* locus.

DNA sequencing. DNA fragments subcloned in M13 vectors were sequenced with the United States Biochemicals (Cleveland, Ohio) Sequenase version 2.0 sequencing kit in accordance with the manufacturer's instructions.

Isolation of genomic DNA and Southern analysis. Cells from 50-ml cultures were treated with lysozyme, lysed with sodium dodecyl sulfate, and treated with proteinase K for 2 h. The lysis mixture was extracted once with phenol, once with phenol-chloroform (1:1), and once with chloroform. The DNA was precipitated with ethanol, spooled out, and redissolved in TE. Genomic DNA (5 μg) was digested with various enzymes, fractionated on a 1% agarose gel, and subsequently transferred to Immobilon-NC (Millipore, Bedford, Mass.) in accordance with the manufacturer's protocol. The membrane was prehybridized, hybridized with DNA probes that had been end labeled with polynucleotide kinase, and subsequently washed with a variety of different salt concentrations and temperatures. The membrane was autoradiographed by exposure to Kodak XRP-1 film.

Nucleotide sequence accession number. The nucleotide sequence has been deposited in GenBank under accession no. U38418.

RESULTS

The subtilin-producing mutant resulting from transformation of *B. subtilis* 168 with DNA from *B. subtilis* ATCC 6633 is called *B. subtilis* LH45 (35). The recombination event that produced LH45 is diagrammed in Fig. 1, in which map A shows the organization of the genes in the *spa* operon as determined by this laboratory and other workers (1, 9, 10, 30–32). A total of nine open reading frames (ORFs) have been identified,

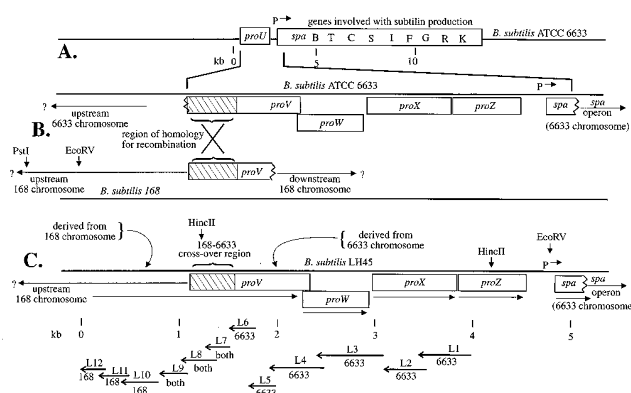

FIG. 1. Organization of *proU* and *spa* operons and recombination between *B. subtilis* 6633 and 168 to form *B. subtilis* LH45. (A) Organization of genes in the *spa* operon of *B. subtilis* 6633, which is a natural subtilin producer. The location of the *proU* operon is shown. (B) The cross-hatched boxes show the putative region of homology between *B. subtilis* 6633 and 168 in which the recombination event occurred (represented by the large X), as established by hybridization experiments described in the text and shown in Fig. 5. The *Eco*RV site shown on the far left was used to generate the 5.3-kb *Eco*RV-*Eco*RV fragment employed for construction of the deletion mutant described in Fig. 6. (C) Organization of the *proU* and *spa* operons in *B. subtilis* LH45, which is a chimera of *B. subtilis* 6633 and 168. The *Eco*RV site on the far right was the other end of the *Eco*RV-*Eco*RV fragment mentioned for panel B. The arrows at the bottom show the sequencing strategy used to obtain the sequence of the *proU* region of LH45 presented in Fig. 2. Each arrow is identified by the synthetic oligo used as a primer to generate the sequence represented by the arrow. Each synthetic oligo was hybridized to genomic DNAs from strains 6633, 168, and LH45 as described in the text and in the legend to Fig. 5. Every oligo gave a hybridization signal with strain LH45. Whether the oligo gave a hybridization signal with strain 6633, 168, or both is indicated below each arrow. L1 to L6 hybridized only to 6633, L7 to L9 hybridized to both 6633 and 168, and L10 to L12 hybridized only to 168.

some lying upstream and others lying downstream of the subtilin structural gene (*spaS*). The sequence of Chung and Hansen showed that a ρ-independent terminator and an ORF were upstream from the *spa* promoter (9). In this work, we extended this sequence further upstream to look for additional ORFs, to determine if there are any that might be associated with subtilin production, to identify the region of recombination between strains 168 and 6633, and to determine the functional role of the conserved region in which the recombination occurred.

Gene sequences upstream from the subtilin operon encode ORFs that are homologous to known ProU proteins. An approximately 4.5-kb sequence extending upstream from the subtilin operon was determined in *B. subtilis* LH45 (Fig. 2). The ORFs identified within this sequence were used as query sequences in the TFASTA program (Genetics Computer Group, Madison, Wis.) to search the GenBank database for homologies. Some excellent homologies to proteins encoded in the osmoregulatory *proU* operon of *E. coli* and *S. typhimurium* were discovered. Three genes have been identified in the *E. coli proU* operon; they are *proV*, *proW*, and *proX*, and they are organized as 5′-*VWX*-3′ (14, 25). The proteins identified as ProV, ProW, and ProX in Fig. 2 are homologous to the *E. coli* ProV, ProW, and ProX proteins, respectively. The protein identified as ProZ in Fig. 2 possesses considerable homology to *E. coli* ProW, and the presence of this fourth *proU*-type gene in the *B. subtilis* operon suggests that *B. subtilis* has a more complex osmoregulatory apparatus than does *E. coli*. These homologies are shown in Fig. 3. The best homology is between *E. coli* ProV and *B. subtilis* ProV, with 35% of the amino acids within a 351-amino-acid overlap being identical. There is a somewhat smaller (29%) identity between the ProW proteins.

FIG. 2. Sequence upstream from the subtilin operon in *B. subtilis* LH45. The 12 sequencing primers that were used for dideoxy sequence analysis are in boldface italics. The locations within the operon are shown in Fig. 1. The ORFs identified as described in the text are shown translated into polypeptide sequences. Each of the identified ORFs has a GGAGG ribosome-binding site (shown underlined) at an appropriate distance upstream from a putative Met initiation codon. The ORFs show considerable similarity to genes present in the *proU* operons of *E. coli* and *S. typhimurium* and accordingly are identified as *proV*, *proW*, *proX*, and *proZ*, respectively. The sequence extending downward from position 3930 was previously published by Chung and Hansen (9) and includes the putative ρ-independent terminator downstream from *proZ* and the promoter for the *spa* operon. The ORF identified as *spaE* is the same as the ORF called *spaB* by Entian and coworkers (28).

The similarity of the putative *Bacillus* ProX protein to *E. coli* ProX is considerably poorer, with only 17% identity obtainable, even when fairly large gaps are allowed. Nevertheless, the ProX proteins are about the same size and there is considerable similarity between nonidentical residues. The similarity of *Bacillus* ProZ to *E. coli* ProW is qualitatively different in that the homologies do not extend throughout the proteins, with the smaller (223 residues) *B. subtilis* ProZ protein being similar to the C-terminal end of the 354-residue *E. coli* ProW protein. The presence of the ProZ ORF may represent a new functionality that is not possessed by the gram-negative *proU* locus.

The fundamental differences in the cell envelopes of gram-negative and gram-positive bacteria raise the possibility of correlative differences in the ProU proteins. For example, the ProX protein in *E. coli* is a periplasmic glycine betaine-binding protein (45) and such proteins are generally soluble periplasmic proteins (17). The relatively low 17% homology between *E. coli* ProX and *B. subtilis* ProX may be a reflection of the fact that *B. subtilis* ProX does not function in a periplasmic environment. To explore this idea, the hydropathic profiles of the two ProX proteins were determined and compared (Fig. 4). Also, the algorithm of Rao and Argos was used to search for transmembrane helices (41). Use of this algorithm showed that *E. coli* ProX has a probable membrane helix corresponding to residues 2 to 22 and *B. subtilis* ProX has a probable membrane helix region corresponding to residues 8 to 28. The probability that *B. subtilis* ProX contains a membrane-spanning region was slightly higher (peak value, 1.3) than that of *E. coli* ProX (peak value, 1.27). The locations of these putative membrane-spanning regions are suited to their functioning to anchor the protein to the cytoplasmic membrane, but if *E. coli* ProX were anchored, it would not be soluble in the way that is characteristic of periplasmic binding proteins (17), so its anchoring seems questionable. On the other hand, it could be important for *B. subtilis* ProX to be anchored, because a gram-positive bacterium does not possess a periplasmic space, and an unanchored binding protein might drift away from the cell, where it probably would not function properly. The hydropathic profiles of the two proteins are not greatly different; the average hydropathy of *E. coli* ProX is −3.21, and that of *B. subtilis* ProX is −3.8. Despite being more hydrophilic on average, the hydrophobic regions of the *B. subtilis* protein are more concentrated in the N-terminal region, suggesting a greater likelihood of its association with the membrane. Little is known about the gram-positive counterparts of periplasmic binding proteins, and this *B. subtilis* ProU system may be suitable for studying these components in a gram-positive organism.

The *proU* operon of *B. subtilis* LH45 is a chimera of the *proU* operon of *B. subtilis* 6633 and the *proU* operon of *B. subtilis* 168. Figure 1 shows how *B. subtilis* LH45 was derived from a double recombination that resulted in incorporation of a fragment from the *B. subtilis* ATCC 6633 chromosome into the *B. subtilis* 168 chromosome. The crossover region shown is on the upstream side of the integrated *spa* operon, and we have determined that the crossover region is within the *proV* gene of the *proU* operon, which means that the *proU* operon in Fig. 2 is a chimera in which the upstream portion is derived from the *B. subtilis* 168 chromosome and the downstream portion is derived from the *B. subtilis* ATCC 6633 chromosome. The location at which recombination occurred was determined in the following way. Since recombination normally requires a region of homology between the sequences that undergo recombination, we reasoned that an appropriate homology must exist between strains 6633 and 168 in the region upstream from the *spa* operon and that if the sequence were determined by "walking" upward, sequence analysis would proceed from within the region derived from strain 6633, across the region of homology, and then into the region derived from strain 168. The sequence analysis was performed by the dideoxy method, with synthetic oligonucleotides (oligos) as primers. The sequencing strategy, shown in Fig. 1, employed a total of 12 oligos (L1 through L12), and the sequence obtained by the use of each successive oligo primer was used to provide the sequence of the next synthetic oligo primer. To determine the crossover

```
A
                              10        20        30        40
bproV               MFADIRKCLENIKGGKKAVNNVNLKIAKGEFICFIGPSGCGKTTTMK
                                        :|::::|  |::||::  ::|  ||:||:|  ::
eproV  IFGEHPQRAFKYIEQGLSKEQILEKTGLSLGVKDASLAIEEGEIFVIMGLSGSGKSTMVR
               20        30        40        50        60        70

        50        60        70            80        90       100
       MINRLIEPSAGKIFIDGENIMEQDPEEL----RRKIGYVFQQIGLFPHMTIQQNISLVPK
       ::||||||:  |:::|||  :|  :  :  :||    |:||:  |||  ::|:||||:  :|:::  :
       LLNRLIEPTRGQVLIDGVDIAKISDAELREVRRKKIAMVFQSFALMPHMTVLDNTAFGME
           80        90       100       110       120       130

          110       120       130       140       150       160
       LLKWPEQHRKERARELLKLVDMGPEYLDRYPHELSGGQQQRIGVLRALAREPPLILMDEP
       |     :::|:|:|  :  |:  |:::  :|  ::||:|||||  :||:|:  ||||  :|  ::||||:
       LAGINAEERREKALDALRQVGLE-NYAHSYPDELSGGMRQRVGLARALAINPDILLMDEA
          140       150       160       170       180       190

          170       180       190       200       210       220
       FGALDPITRDSLQEEFKKLQKTLHKTIVFVTHDMDEAIKLADRIVILKAGEIVQVGTPDD
       |:||||::|:::|:|:  |||  :  ::||||::||:||||::::|||:|:::||:|||||||:
       FSALDPLIRTEMQDELVKLQAKHQRTIVFISHDLDEAMRIGDRIAIMQNGEVVQVGTPDE
           200       210       220       230       240       250

          230       240       250       260       270       280
       ILRNPADEFVEEFIGKERLIQSSSPDVERVDQIMNTKPVTITADKTLSEAIQLMRQERVD
       ||:|||:::|  :|:      :  |   |:  :  :::    :   :   |::  :  ::|::|:::|   :
       ILNNPANDYVRTFFRGVDISQVFSA--KDIARRTPNGLIRKTPGFGPRSALKLLQDEDRE
           260       270       280       290       300

          290       300       310       320       330       340
       SLLVVDGEHVLQGYVDVEIIDQCRKKANLVGEVLHEDLYTVLGGTLLRDTVRKILKRGIK
          |::  ::  :  |  |:::  ::    ::::  ::::|  ::    :|  :  |  |::  ::::  :
       YGYVIERGNKFVGAVSIDSLKTALTQQQGLDAALIDAPLAVDAQTPLSELLSHVGQAPCA
     310       320       330       340       350       360

          350       360       370       380       390       400
       YVPVVDGNRRLIGIVTRASLVDIVYDSSGGKKISSRYCHREVTSHASYYSIFTNQRGRTP
        |||||::::  :||::::  |:
       -VPVVDEDQQYVGIISKGMLLRALDREGVNNG
      370       380       390       400

B
                          10        20        30        40        50
bproW                MHHIIQFLQTNGGELLYKTYEHITISLIAVILGVLVAVPLGVVLTRMKKGAG
                                            :::  |:|:::  :::::|||:  |:|   ::|
eproW  QISGVGMGVATLVSLIAIGAIGAWSQAMVTLALVLTALLFCIVIGLPLGIWLARSPRAAK
      120       130       140       150       160       170

             60        70        80        90       100       110
       TIIGIVNIIQTLPSPDSRHIFIPLLGVGKVPAIVALFFYSVLPILRNTYTGIRGVNKNLL
       :|  :::  :||  |:        ::  |:|:|:||::|:  :::::  ||:|  |   ||:  |   :|:
       IIRPLLDAMQTTPAFVYLVPIVMLFGIGNVPGVVVTIIFALPPIIRLTILGINQVPADLI
      180       190       200       210       220       230

            120       130       140       150       160       170
       ESGKGIGMTPAEQVRLVDVPLAAPVIMAGIRTSTIYLIGWATLASFIGGGGLGDYIFIGL
       |:::::|  :|  :  :   |::|||  |:||||::  :  :   ::  :::||:|:  ||||:  ::  |:
       EASRSFGASPRQMLFKVQLPLAMPTIMAGVNQTLMLALSMVVIASMIAVGGLGQMVLRGI
      240       250       260       270       280       290

            180       190       200       210
       NLYQPEYIIGGAVPVTILAIVIDYVLAVTERKLTPAGMQRLKEVSX
       :  :  :  :  |:|  ::||||::|  :  ::::|:  :  |  :|
       GRLDMGLATVGGVGIVILAIILDRLTQAVGRDSRSRGNRRWYTTGPVGLLTRPFIKX
      300       310       320       330       340       350

C
                     .         .         .         .         .
bproX  1 MKRKYLKWMIGLTLAAMLPLSGCSLPGLSAASDQTIKIGAQSMSESEIIA 50
                :: : :. |    :|. .::: ::.: |:. .. .:.|..: .
eproX  1 .....MRHSVLFATAFATLISTQTFAADLPGKGITVNPVQSTITEETFQT 45

                     .         .         .         .         .
      51 SMLGQLIEHHTDLKTTTIKNLGSNAVQQQALMNREIDIAATRYTGDALTG 100
          ::::. :|.                : .|.... :: ::::...    .:..:|:
      46 LLVSRALEKL............GYTVNKPSEVDYNVGYTSLASGDATFTA 83

                     .         .         .         .         .
     101 TLRMEPEKDPEKALALTQREFKKRYDLKWYDSYGFDNTYAFTVSKKLADQ 150
          . : . ..: .| : ..: ::.      : :. :.. :: :.|| |||
      84 VNWTPLHDNMYEAAGGDKKFYRE.......GVFVNGAAQGYLIDKKTADQ 126

                     .         .         .         .         .
     151 YHLENVSDVK..KWAPQLKLGVDKVLD...........EAQGERLSRFY. 186
          |.:.|:.::|  | |. :. ..|   |           :| ...|. :
     127 YKITNIAQLKDPKIAKLFDTNGDGKADLTGCNPGWGCEGAINHQLAAYEL 176

                     .         .         .         .         .
     187 ........ENYGMTFSGT............YPMQIGLVYDAVKSGKMDIV 216
                 :||:  :.:|             |.    :| :.:|.|| |:|
     177 TNTVTHNQGNYAAMMADTISRYKEGKPVFYYTWTPYWVSNELKPGK.DVV 225

                     .         .         .         .         .
     217 ...LAYSTDGRIKSYGLKMLKDDKQFFPPYDCSPVVPEQVLKEH...... 257
             :::|. .  |. : | |.:: .:  | .. .:|::..: |.
     226 WLQVPFSALPGDKNADTK.LPNGANYGFPVSTMHIVANKAWAEKNPAAAK 274

                     .         .         .         .         .
     258 .......PELEGTIQKMIGKIDTATMQELNYEVDGNLKEPSVVAKAIFRK 300
                 |  : . |. | . :.|. .::. .||| :|..    .: ...
     275 LFAIMQLPVADINAQNAIMHDGKASEGDIQGHVDGWIKAHQQQFDGWVNE 324

                     .
     301 APLLRIVKGGRSQ* 314
         |    .
     325 ALAAQK*....... 331

D
10        20        30        40        50
bproZ     MNVLEQLMTYYAQNGSYVMDEFGRHFLMSVYGVLFAAVVGVPVGILIAHYRRLSAWV
                                          : : ::||  |:|:|:||  :|: :|  :  :
eproW  SGVGMGVATLVSLIAIGAIGAWSQAMVTLALVLTALLFCIVIGLPLGIWLARSPRAAKII
              130       140       150       160       170       180

        60        70        80        90       100       110
       FAVTNVIQTIPALAMLAVLMLVMGLGANTVIISLFLYSLLPIIRNTYTGIVSIEHAYLES
        :: :::||:||:: |: :::::|:|: : ::  ::::| |||| |  ||  :  : :|:
       RPLLDAMQTTPAFVYLVPIVMLFGIGNVPGVVVTIIFALPPIIRLTILGINQVPADLIEA
              190       200       210       220       230       240

       120       130       140       150       160       170
       GKAMGMTKFQVLRMVELPLALSVIMAGLRTALVIAIGITAIGTFVGAGGLGDMIVRGSNA
       ::::| :  |:| :|:||||:::||||:: :|::|:::::|::::::||||:|::||  :
       SRSFGASPRQMLFKVQLPLAMPTIMAGVNQTLMLALSMVVIASMIAVGGLGQMVLRGI-G
              250       260       270       280       290

       180       190       200       210       220
       TNGTAIILAGATPTAVMAIGADLIMAWIERFLNPVKQKSRRKVISV
         : ::  :|:: ::::||  | : : ::|
       RLDMGLATVGGVGIVILAIILDRLTQAVGRDSRSRGNRRWYTTGPVGLLTRPFIKX
     300       310       320       330       340       350
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FIG. 3. Homologies between proteins encoded in the *E. coli proU* operon and the *B. subtilis proU* operon. The sequences of the proteins encoded in the *E. coli proU* operon (25) are compared to the corresponding deduced proteins from the putative *proU* operon from *B. subtilis* LH45. Identical amino acid residues are connected with dashes, and similar amino acid residues are connected with dots. (A) Similarity between *E. coli* ProV and the deduced sequence of *B. subtilis* ProV. There is 35% identity within a 351-amino-acid overlap. (B) Similarity between *E. coli* ProW and the deduced sequence of *B. subtilis* ProW. There is 29.5% identity in a 190-amino-acid overlap. (C) Similarity between *E. coli* ProX and the deduced sequence of *B. subtilis* ProX. There is only 17% identity, but this extends throughout both proteins, which are about the same size. (D) Similarity between *E. coli* ProW and *B. subtilis* ProZ. There is a 29.9% identity in a 291-amino-acid overlap.

region, each sequencing oligo was labeled and used to probe Southern blots of total chromosomal DNAs from strains LH45, 168, and 6633 that had been subjected to restriction enzymes. What we expected to see was a hybridization signal to LH45 from all of the probes, a hybridization signal to 6633 when the probe sequence was present in 6633, and a hybridization signal to 168 when the probe sequence was present in 168. The region of crossover homology should be common to all three strains. Figure 5 shows the results of hybridization experiments with probes L6 to L10. All of the probes hybridized to LH45, as expected. Probes L1 through L6 hybridized only to DNA from 6633, probes L7 through L9 hybridized to both 6633 and 168, and probes L10 through L12 hybridized

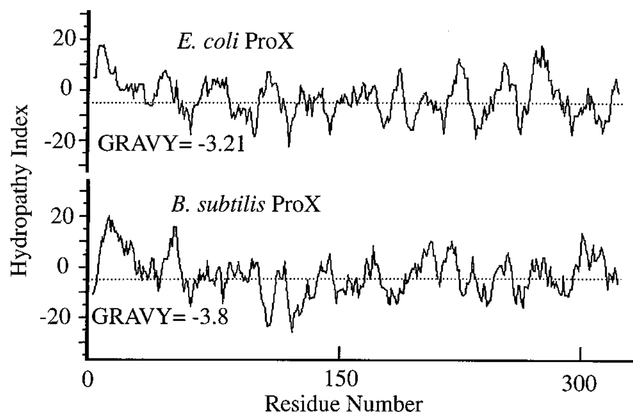

FIG. 4. Hydropathy profiles of *E. coli* ProX and *B. subtilis* ProX. The hydrophobicity of the protein is represented as a hydropathicity index, computed by using the method of Kyte and Doolittle (33), which is plotted against the residue number; the dotted line is the grand average of known proteins. GRAVY is the average hydropathy of the entire protein. The locations of putative integral membrane regions were determined by the method of Rao and Argos (41).

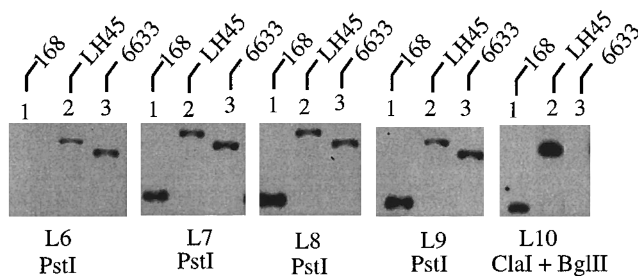

FIG. 5. Hybridization of probes to genomic DNAs of strains 6633, 168, and LH45. The sequencing primers shown in Fig. 1 were hybridized to Southern blots of genomic DNAs isolated from *B. subtilis* 6633, 168, and LH45 and digested with restriction enzymes. Each primer was tested, and the pattern of hybridization signals obtained is summarized in Fig. 1 and in the legend to Fig. 1. Shown here are hybridization data obtained with probes L6 to L10, which is the region that encompasses the region of homology at which recombination between strains 6633 and 168 occurred, to generate strain LH45. L6 hybridized to 6633 and LH45 but not to 168. L7 to L9 hybridized to all three strains, showing that considerable homology exists within this region. L10 hybridized with 168 and LH45 and not with 6633. L1 to L5 hybridized only with LH45 and 6633 (data not shown), and L11 and L12 hybridized only with LH45 and 168 (data not shown). The restriction enzyme(s) used to digest each genomic DNA is indicated below the respective autoradiogram.

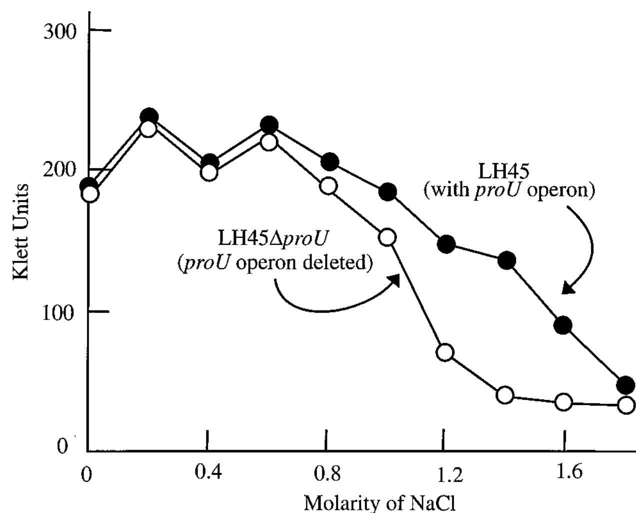

FIG. 6. Effect of high osmolarity on the growth of LH45 and LH45Δ*proU*. Cultures (5 ml) were incubated for 15 h in defined medium (supplemented with PAB) at different concentrations of NaCl. The cell density attained after 15 h is plotted against the NaCl concentration of the medium. Strain LH45 contains an intact *proU* operon, whereas LH45Δ*proU* has most of the *proU* operon deleted, as described in the text.

only to strain 168 (not all results are shown). We concluded from these results that the crossover occurred in the L7 to L9 region, which is about 500 bp long; this is long enough to support homologous recombination. It was this result that allowed us to conclude that the *proU* region that we sequenced in strain LH45 is a chimera in which the downstream portion is contributed by strain 6633 and the upstream portion is contributed by strain 168; the regions contributed by both strains are shown in Fig. 1. Moreover, since the region of homology to support recombination occurs within the *proV* gene, the ProV protein that is encoded by the chimeric LH45 *proV* gene is a chimeric protein in which the N-terminal portion is contributed by the 168 *proV* gene and the C-terminal portion is contributed by the 6633 *proV* gene. Because this sequence is a chimera, it does not provide us with the complete organization of the actual *proU* operon from either strain 6633 or 168. However, experiments described below showed that the chimeric *proU* locus is fully functional in the ability to confer osmotolerance in the presence of glycine betaine as an osmoprotectant. This argues that the chimeric ProV protein is a fully functional homolog of the corresponding ProV proteins in strains 168 and 6633. Determining the sequences of the *proU* loci in either strain would be a straightforward process of extending the sequence analysis in those two strains by using the chimeric sequence as a starting point for each strain.

Deletion of the *proU* operon from *B. subtilis* LH45 reduces its ability to grow at high osmolarity. The chimeric *proU* operon in strain LH45 suggests that the strains from which the composite parts were derived (168 and 6633) each contained functional *proU* operons. The ability of the chimeric *proU* operon in strain LH45 to provide osmoprotection was established by constructing a mutant of LH45 in which the *Hin*cII fragment shown in Fig. 1 (which contains most of the chimeric *proU* region) was deleted as described in Materials and Methods. Southern hybridization analyses of restriction digests of the deletion mutants were used to verify that the deletion had occurred as intended. The deletion mutant is called LH45Δ*proU*. The mechanism by which *proU* confers osmotolerance is by uptake of osmoprotectants, such as proline or glycine betaine. In an initial experiment, LH45 and LH45Δ *proU* cells were grown for 15 h in the defined medium supplemented with 0.1% PAB (which contains low levels of glycine betaine and other osmoprotectants) and different concentrations of NaCl. At the end of the 15-h growth period, the density of the cells was measured and plotted against the NaCl concentration as shown in Fig. 6. Significant differences in the cell density attained after 15 h were observed in the 1.2 to 1.6 M range of NaCl, with the *proU* deletion mutant growing less well, as expected if it had been unable to import osmoprotectants. To test this further, growth curves were determined in which cells were grown in the defined medium (no PAB), in the presence or absence of glycine betaine as an osmoprotectant, and in the presence or absence of high osmolarity (1.2 M NaCl). The growth curves are shown in Fig. 7. LH45 and LH45Δ*proU* grew equally well at low osmolarity in the absence of glycine betaine. This was expected, since at low osmolarity

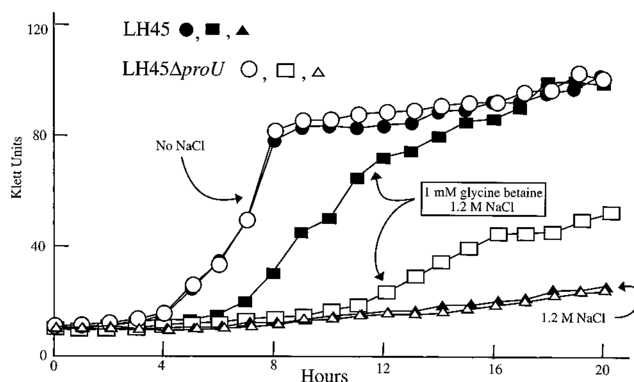

FIG. 7. Loss of glycine betaine-dependent osmoprotection in cells with deletion of the *proU* operon. Growth curves of strains LH45 and LH45Δ*proU* at low osmolarity (circles), high osmolarity (triangles), and high osmolarity in the presence of glycine betaine (squares). Experiments were performed with 25-ml volumes of the defined medium (with supplemental amino acids, no supplemental PAB) in 125-ml sidearm flasks. Cell density (in Klett units) is plotted against growth time. Strain LH45 (filled symbols) contains the chimeric *proU* operon as defined in Fig. 1, and strain LH45Δ*proU* (open symbols) has most of the *proU* operon deleted.

no osmoprotection is needed. In the presence of 1.2 M NaCl and without glycine betaine, LH45 and LH45Δ*proU* both grew extremely poorly. This shows that the defined medium contains nothing that either strain can employ as an osmoprotectant and that the presence of the *proU* operon in LH45 confers no advantage under such circumstances. The next set of growth curves employed both glycine betaine and high osmolarity. Since a normal *proU* operon encodes a system for the uptake of glycine betaine, a cell with a functional *proU* operon should show osmoprotection in the presence of glycine betaine. Figure 7 demonstrates that LH45, which contains the chimeric *proU* operon as defined in Fig. 1, grew quite well in the presence of 1.2 M NaCl when the medium was supplemented with 1 mM glycine betaine. The only effect of high osmolarity was to delay the onset of logarithmic growth, and once logarithmic growth was under way, the growth rate of LH45 at high osmolarity (when glycine betaine was available) was very similar to its growth rate at low osmolarity, and the same cell density at the stationary phase was attained for both low and high osmolarities. Moreover, examination of stationary-phase cells by phase-contrast microscopy showed no differences in morphology, nor were there any signs of cell lysis at low or high osmolarity when the cells were able to protect themselves by uptake of glycine betaine. In contrast, when LH45Δ*proU* cells were grown at high osmolarity, the presence of glycine betaine provided relatively little benefit, which is expected if this *proU* deletion mutant cannot import the glycine betaine osmoprotectant. The time prior to onset of significant growth was extended, and this was followed by relatively little growth. Examination of the cells after 20 h by phase-contrast microscopy revealed cells with normal morphology and no signs of lysis. Therefore, the low cell density was not due to abnormal cell size or lysis but to the fact that they grew very slowly. The fact that strain LH45Δ*proU* grew slightly better in the presence of glycine betaine despite its *proU* region having been deleted can be explained by the presence of other glycine betaine transport systems. For example, there is evidence that *B. subtilis* possesses at least two glycine betaine import systems (5). If this is true, the data in Fig. 7 suggests that the *proU* import system is the most important one, since its disruption shows a dramatic reduction in the ability of the cell to utilize glycine betaine as an osmoprotectant.

DISCUSSION

The ability of cells to respond to osmotic stress is widely distributed among highly diverged organisms, including bacteria, plants, and animals (46). Although osmoregulation has been extensively studied in gram-negative bacteria such as *E. coli* and *S. typhimurium*, there are excellent reasons for extending these studies to gram-positive bacteria. *Staphylococcus aureus* is among the most osmotolerant of the nonhalophilic eubacteria and can grow in NaCl at concentrations of up to 3.5 M (44). *B. subtilis* is a soil-dwelling organism and is, accordingly, subjected to frequent osmotic challenges imposed by the drying and wetting of its habitat (5). One would therefore expect that exploration of osmoregulatory mechanisms in gram-positive bacteria will lead to better understanding of the strategies that cells use to achieve osmotolerance. Our discovery of a *proU*-type locus in *B. subtilis* is therefore an important step to acquiring this knowledge.

The presence of the *proU* locus in both gram-negative and gram-positive bacteria indicates that this locus is of ancient origin and evolved before the split of prokaryotes into the two kingdoms. This is not surprising in view of the fact that the ability to respond to changes in the osmotic environment would have been advantageous to primitive life forms. If the *proU* locus was present prior to the evolutionary split between gram-positive and gram-negative prokaryotes, it has had an opportunity to evolve among gram-positive bacteria since that time. One of our purposes in carrying out this work was to learn about the recombination event that occurred during the conversion of *B. subtilis* 168 to a subtilin producer by transformation with DNA from *B. subtilis* 6633. Prior to this work, *B. subtilis* 6633 had been completely uncharacterized genetically, so the relationship between the 168 genome and the 6633 genome was completely unknown. Our studies within the *proU* locus have established both similarities and differences between these genomes. The genomes are similar in that they both possess a *proU* locus, there is sufficient homology within the *proV* gene to support homologous recombination, and the hybridization analysis with synthetic oligo probes shows the presence of an about 500-bp region of very good homology within which the recombination event occurred. However, outside this stretch of homology, in both the upstream and downstream directions, the homology drops to the point that there is no cross-hybridization of the oligo probes between 6633 and 168. This shows that these two strains have drifted apart considerably at the nucleotide level. However, it appears that conservation of function at the protein level is much better. The ability of LH45 (which is derived from strain 168) to display osmoprotection despite the fact that it contains a 168-6633 chimera of the *proV* gene, as well as the 6633 *proW*, *proX*, and *proZ* genes, shows that the proteins that are encoded by these genes are able to cooperate sufficiently well to provide normal osmoprotection.

An interesting aspect of the *proU* locus in *B. subtilis* is that it contains a fourth cistron, which we call *proZ*, that is lacking in the *proU* operon of either *E. coli* or *S. typhimurium*. The ProZ protein that this encodes shows significant homology to the ProW protein, although the ProZ protein contains only 223 residues, compared with the 354-residue ProW protein, with the homology being within the C-terminal portion of ProW. ProW possesses characteristics of an integral membrane protein that is involved in transport (25). A search of the databases with ProZ as a query sequence did not provide any insights beyond this. We therefore speculate that ProZ participates in osmoregulation and complements the functions provided by the VWX proteins. One possibility is that it is involved in the import of one or more additional osmoprotectants, thus increasing the range of osmoprotectants that can be employed by the cell.

Finally, we note that the genes upstream from the *spa* operon correspond to known *proU* genes that participate in osmoregulation and that this same *proU* region is present in both subtilin-producing strain 6633 and nonproducing strain 168. Because the *proU* region is present in 168, we conclude that the *proU* genes are not involved in subtilin biosynthesis, that the promoter of the *spa* operon constitutes the beginning of the region of the 6633 chromosome that is involved in subtilin biosynthesis, and that all of the subtilin biosynthesis genes therefore lie downstream from this promoter.

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