Molecular analysis of an operon in *Bacillus subtilis* encoding a novel ABC transporter with a role in exoprotein production, sporulation and competence

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The levels of exoamylase and other exoenzymes of *Bacillus subtilis* are pleiotropically decreased by the *ecs-26* (*prs-26*) and *ecs-13* (*prs-13*) mutations. These mutations also cause a competence- and sporulation-deficient phenotype. In the present work, the *ecs* locus, which has been defined by the *ecs-26* and *ecs-13* mutations, was cloned and sequenced. Sequence analysis revealed a putative operon of three ORFs (*ecsA*, *ecsB* and *ecsC*). *ecsA* can encode a putative polypeptide of 248 amino acid residues containing an ATP-binding site. The polypeptide shows about 30% sequence similarity with the ATP-binding components of numerous membrane transporters of the ABC-type (ATP-binding cassette transporters or traffic ATPases). The *ecs-26* mutation was found to result from a transition of one base pair changing the glycine₁₆₄ of *EcsA* to a glutamic acid residue in the vicinity of the putative ATP-binding pocket. *ecsB* was predicted to encode a hydrophobic protein with six membrane-spanning helices in a pattern found in other hydrophobic components of ABC transporters. The properties deduced for the *ecsA* and *ecsB* gene products are consistent with the interpretation that *ecs* encodes a novel ABC-type membrane transporter of *B. subtilis*. The third ORF, *ecsC*, can encode a putative polypeptide of 237 amino acid residues. The polypeptide does not resemble components of ABC transporters.

Keywords: *Bacillus subtilis*, *ecs* operon, ABC transporter, secreted proteins

INTRODUCTION

The ATP-binding cassette (ABC) transporters (Hyde *et al.*, 1990), alternatively called traffic ATPases (Ames *et al.*, 1990), form a large family of membrane transporter systems or permeases found in both prokaryotic and eukaryotic cells (for reviews see Higgins, 1992, and Doige & Ames, 1993). ABC transporters function in the import or export of a wide range of products such as proteins, peptides, polysaccharides, vitamins and drugs, utilizing ATP as the source of energy. In addition to the import or export of molecules, the function of an ABC transporter may be connected to the regulation of cellular functions

and signal transduction, such as the induction of sporulation, which is associated with oligopeptide transport in *Bacillus subtilis* (Rudner *et al.*, 1991; Perego *et al.*, 1991).

ABC transporters have a complex structure. In general, each transporter consists of two ATP-binding components and two highly hydrophobic components. Stoichiometrically, the different components are either in the form of homo- or heterodimers. The ATP-binding components are hydrophilic proteins which hydrolyse ATP to energize the transport. The ATP-binding components of all ABC transporters display extensive sequence homology that extends beyond the ATP-binding motifs (Higgins *et al.*, 1986; Ames *et al.*, 1990). The ATPase components are associated with the integral hydrophobic membrane protein components, which are presumed to form a transport channel (Kerppola *et al.*, 1991; Higgins, 1992). In addition to the membrane-bound complex, bacterial transporters often contain other

Abbreviation: ABC, ATP-binding cassette.

The EMBL accession number for the sequence reported in this paper is X87807.

components. These include substrate-binding proteins in the case of several importers. The binding proteins are periplasmic in Gram-negative bacteria (Doige & Ames, 1993) and peripheral lipoproteins of the cytoplasmic membrane in Gram-positive bacteria (Gilson *et al.*, 1988; Alloing *et al.*, 1990; Mathiopoulos *et al.*, 1991; Perego *et al.*, 1991; Rudner *et al.*, 1991). Several bacterial exporters have been found to contain accessory membrane-bound proteins (Fath & Kolter, 1993). The components of a bacterial transporter are often separate proteins encoded by one multicistronic operon, but may also be different domains of a single polypeptide, which is the structural arrangement found in eukaryotic ABC transporters (Higgins, 1992).

We have previously applied a mutant screen on *B. subtilis* to obtain non-conditional mutations affecting cellular components of the secretion machinery or the regulation of genes encoding secreted proteins. We have mutagenized a strain overexpressing secreted α -amylase from the *amyE* gene of *Bacillus amyloliquefaciens*. Mutants have been isolated by screening for decreased levels of secreted α -amylase and the identified mutations have been mapped in four different chromosomal loci (Kontinen & Sarvas, 1988).

The *ecs* (effect on exoproteins, defect in competence and sporulation, previously called *prsT*) locus was defined by two of these mutations, *ecs-26* and *ecs-13* (*prs-26* and *prs-13* in Kontinen & Sarvas, 1988). The locus maps close to *glyB*, on the other side of this marker than the *prsA* gene, which encodes a cellular component of the secretion machinery and was found in the same mutant screen as the *ecs* locus (Kontinen & Sarvas, 1993). Both *ecs* mutants have a similar, pleiotropic phenotype. The level of the overexpressed heterologous α -amylase was decreased to about 10% of the wild-type level in the culture medium. The secreted amounts of exoproteins expressed at a much lower level were also decreased by the *ecs* mutations, although only moderately. Such proteins are endogenous proteases, α -amylase of *B. subtilis* and a heterologous β -lactamase. In addition, the mutants are not competent for transformation and their sporulation frequency is only 1% of that of the wild-type (Kontinen & Sarvas, 1988).

The aim of the present study was to clone and sequence the *ecs* locus, and to characterize the *ecs-26* mutation causing the pleiotropic phenotype.

METHODS

Bacterial strains, plasmids, growth conditions, and the plate assay of α -amylase. Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, strains were grown in L-broth, on L-agar or SMS-agar (Spizizen minimal medium; Anagnostopoulos & Spizizen, 1961) plates containing glucose (5 mg ml⁻¹), and supplemented with essential amino acids. Ampicillin (100 μ g ml⁻¹) or kanamycin (10 μ g ml⁻¹) were added as indicated. Secretion of α -amylase was assayed with the plate method as described in Kontinen & Sarvas (1988). The colonies were grown on L-agar plates containing 5% (w/v) starch and the diameter of the clear halo around colonies was determined.

DNA techniques. Basic DNA techniques were those described in Sambrook *et al.* (1989). *B. subtilis* transformation was done as described in Gryczan *et al.* (1978). Chromosomal DNA was isolated from *B. subtilis* as described in Marmur (1961). For DNA sequencing, the dideoxy chain termination method of Sanger *et al.* (1977) and synthetic oligonucleotide primers were used.

Cloning of *ecs* locus. Since the *ecs-26* mutant is not competent for transformation, we decided to clone the *ecs-26* mutant locus to facilitate identification and analysis of recombinant clones. A lambda library of chromosomal fragments of the *ecs-26* mutant (IH6821) was constructed as described in Kontinen *et al.* (1991) in the LambdaGEM-11 vector (Promega). The chromosomal fragments were produced by partial *Sau3A* digestion. The lambda library was screened by Southern hybridization with ³²P-labelled plasmid pKTH3208 as a probe, which carries an approximately 2.3 kb insert of *B. subtilis* DNA and contains the *glyB133* marker. The *ecs-26* mutation is closely linked to the *glyB133* marker (Kontinen & Sarvas, 1988). The lambda clones which hybridized with the probe were tested for the presence of the *ecs-26* mutation as follows. A *glyB133* mutant (IH6531) carrying plasmid pKTH10, and therefore secreting large amounts of α -amylase, was transformed with recombinant λ DNA and transformants were selected for the ability to grow without glycine (*gly*⁺). The presence of the *ecs-26* mutation in the *gly*⁺ transformants was tested by streaking single colonies onto starch plates (Kontinen & Sarvas, 1988). The presence of the *ecs-26* mutation was identified by small haloes on those plates.

Computer analysis. GCG programs (Wisconsin Sequence Analysis Package, University of Wisconsin) were used for sequence analysis.

RESULTS AND DISCUSSION

Cloning, subcloning and mapping of the *ecs* locus

The *ecs* locus was cloned by constructing a LambdaGEM-11 genome library of the *ecs-26* mutant and screening for colonies which contained the *glyB*⁺ marker. A lambda clone (λ K10) containing an insert with the *glyB133* marker was isolated and tested for the presence of the *ecs-26* mutation as described (see Methods). About 60% of the *gly*⁺ transformants obtained were found to secrete α -amylase at the low level characteristic of the *ecs-26* mutant, indicating that the chromosomal insert in λ K10 contains both the *glyB133* and *ecs-26* markers.

Fig. 1. Partial restriction map of the 2.3 kb *SacI* fragment of pKTH3234 and inserts of the derivative plasmids (pKTH3243, pKTH3245, pKTH3254 and pKTH3255). The presence (+) or absence (-) of *ecs-26* or *glyB133*⁺ is shown in the right panel.

Fig. 2. Partial restriction map of a 5.5 kb chromosomal DNA fragment from the *ecs-26* mutant. The inserts of the subclones pKTH3234, pKTH3270 and pKTH3232 used for sequencing, and the orientation of the ORFs in the *ecs* operon are shown. The 3223 bp segment between the *EcoRI* and *PstI* sites indicated with asterisks was sequenced. The *ecs-26* mutation is located at the *XbaI* site.

The insert of λ K10 spans about 12 kb of the *B. subtilis* chromosome. This insert was digested with *SacI* and the resulting five fragments (4.5 kb, 3.2 kb, 2.3 kb, 1.2 kb and 0.4 kb) were subcloned into pGEM4Z. The subclones were tested for the presence of the *ecs-26* mutation by conjugation (co-transformation with plasmid pKTH3211 carrying the *glyB*⁺ marker). Subclone plasmids were introduced into strain IH6531 (*glyB*¹³³, pKTH10). *gly*⁺ transformants were tested for the presence of the *ecs-26* marker by screening for decreased secretion of α -amylase in the plate assay. Plasmid pKTH3234, which carries the 2.3 kb *SacI* fragment of λ K10, was found to contain the *ecs-26* marker. No transformants of the desired phenotype (low α -amylase secretion) were found with other subclone plasmids. Subsequently, we found that pKTH3234 transformed IH6531 to *gly*⁺ also in the absence of pKTH3211 (*glyB*⁺) DNA. About 20% of the *gly*⁺ transformants carried the *ecs-26* mutation. The 2.3 kb *SacI* fragment thus contains both the *ecs-26* and the *glyB*¹³³ site. Restriction fragments of this fragment were further subcloned into pGEM4Z and analysed for the presence of *ecs-26* as above. The *ecs-26* mutation was thus localized in the 200 bp *XbaI*–*SacI* subfragment (Fig. 1).

Nucleotide sequence of *ecs*

We used three plasmid subclones obtained from λ K10 (pKTH3234, pKTH3270 and pKTH3232) as templates, and sequenced a 3223 bp *EcoRI*–*PstI* segment containing the *ecs-26* mutation (Fig. 2) from both strands. The sequence is shown in Fig. 3.

Sequence analysis revealed that the *ecs* locus consists of three ORFs, *ecsA*, *ecsB* and *ecsC*, in an arrangement indicative of an operon (Figs 2 and 3). A putative promoter (Fig. 3) was found upstream of *ecsA*, with the sequence TTGATA-17bp-TTTTAT resembling the consensus promoter recognized by the δ^A form of RNA polymerase (TTGACA-17bp-TATAAT; Moran *et al.*, 1982). There is a putative rho-independent transcription terminator with a GC-rich palindromic sequence, and a run of T residues downstream of *ecsC*. Each ORF is preceded by a putative ribosome-binding site (RBS); the two 3' terminal codons of *ecsA* overlap with the 5' terminal codons of *ecsB*. A short intercistronic region is present between *ecsB* and *ecsC*. No other ORFs were

identified, and translational stop codons are present in all reading frames in the sequences downstream and upstream of the putative operon.

To pinpoint the *ecs-26* mutation, we studied a 4.5 kb *SacI* fragment derived from the wild-type chromosome. It contained the *glyB*¹³³ marker and had previously been subcloned from the λ K3 clone (Table 1) into pGEM4Z resulting in pKTH3267. The insert in pKTH3267 was characterized by restriction and sequence analysis. Sequence comparison revealed that the *ecs-26* mutation is a transition of G₇₃₈ to A₇₃₈. The mutation generated an *XbaI* restriction site and will result in Gly₁₆₄ to Glu₁₆₄ substitution in the putative EcsA polypeptide.

EcsA shows sequence similarity to ABC transporters

The *ecsA* gene can encode a putative polypeptide of 248 amino acids. Comparison of the deduced amino acid sequence with proteins in the Swiss-Prot database showed about 30% sequence homology to a large number of ATP-binding proteins, which all belong to the superfamily of ABC transporters.

Sequence comparison shows that EcsA displays similarities with ATP-binding components of ABC-type transporters. The sequence alignment with three well-characterized ATP-binding proteins is shown in Fig. 4. First, the two motifs characteristic of ATP-binding proteins (Walker motifs I and II; Walker *et al.*, 1982) are present. In addition, between the Walker motifs I and II, EcsA contains a glutamine- and glycine-rich motif (consensus L/FSGGQQ/R/KQR) that is well-conserved in the ATP-binding components of ABC-transporters (Ames *et al.*, 1992). Homology that extends beyond the ATP-binding motifs was observed between EcsA and ABC transporters in the region of about 200 amino acid residues which also contains the putative motifs of the ATP-binding site. Such homology has not been observed between other ATP-binding proteins (Higgins *et al.*, 1986; Hyde *et al.*, 1990). Hydropathy analysis (Fig. 5a) showed that EcsA is hydrophilic and has no evident membrane-spanning segments. This is a property shared by ATP-binding components of many bacterial ABC-transport systems in spite of their tight membrane-association (Doige & Ames, 1993).

AATTCATCAATTAGTGAAGAGAAATGGTTTGAGCATTGGACATGTTTATTTTCTATCCAACGTGTTTCTAAGACGCTTTT 80
 GACAAACACCTCATTTTTCAGTGAATACTGCTTTTCTCTCAACAATTTTATGATGTCAGAACCCCTGCTCTTTATGACAG 160
 -35 -10
 CAAATTCATCATTTTGTAAAAGGGAATTGTTTCAAATCAGGCCCTTGTTTGTGGTACTATGAAGAAAAACACTATAAGGGG 240
 AGAAACTATGCTCTGCTATCGGTAAAGACCTGACCGCGGATATACAAGGAATCCGGTTTAAAAAACGTATCAATTCA 320
 ecsA-> M S L L S V K D L T G G Y T R N P V L K N V S F T
 CCCTTGAACCGAATCAAATTGTCGGCTTAATCGGGCTGAATGGTGGTGAAGTACAACAAATCAGACATCATCGGG 400
 L E P N Q I V G L I G L N G A G K S T T I R H I I G
 CTGATGGACCGCATAAAGGTTCAATCGAATTAAACGGTAAACGTTTGCCTGAGGATCCGGAAGGCTACCGTTTCACAATT 480
 L M D P H K G S I E L N G K T F A E D P E G Y R S Q F
 TACCTATATACCTGAACACCTGTTTATACGAAGAATTGACGCTGATGGAGCATCTTGAACTAACAGCCATGGCATATG 560
 T Y I P E T P V L Y E E L T L M E H L E L T A M A Y G
 GACTGTCAAAGAAACGATGGAGAAAGGCTGCTTCCGCTACTAAAGGAATTCGGAATGGAAGAGGCTGAAGTGGTTC 640
 L S K E T M E K R L P P L L K E F R M E K R L K W F
 CCGGCCCATTTTCTAAAGGAATGAAGCAGAAGTTATGATTGTCGCATTTTGGCAGAGCCTGCGCTCTACATTAT 720
 P A H F S K G M K Q K V M I M C A F L A E P A L Y I I
 TGATGGACCTTTTCTAGGCTTGATCCGCTTGCCTTAACCGCGCTGCTTGAACGGATGAATGAACGAAAAAGCGGGG 800
 D E P L L G L D P L A I N A L L E R M N E A K K G G A
 CGAGCGTCTGATGTCAACACACATTTGGCAACGGCAGAAGCTATTGCGATTGCTTTATTATTATACATAACGGCGAG 880
 S V G L M S T H I L A T A E R Y C D S F I I L H N G E
 GTGGCGCGCGGACGCTGTCAGAGCTCAGAGAGCAGTTTGAAGTGAAGGACGCGGCGCTGGACGATTGTCATCTTGA 960
 V R A R G T L S E L R E Q F G M K D A A L D D L Y L E
 GCTTACAAAGGAAGACGCTGGCCATGAATAATGCTTATTTGGCAGTCGCGGCTGCAGGAGCATATCAAGAAACA 1040
 L T K E D A G H E -
 ecsB-> M N N M L D I W Q S R L Q E H I K E T
 AGAACATACATGAATATATGCTCAACGATCACCCTGCTCATTTTGGATTTTCTAGCGGGCGCTGCAAGCTGGTA 1120
 R T Y M L N D H L V I V L I F F L A G A A S W Y
 CAGCAAAATGGATACGGGACATTCCTGCTTTCGGTGGTGGCGGCTGCTTTTCCGCTCGTATTGACAA 1200
 S K W I R D I P A H F P S F W V M A V L F S L V L T S
 GCTCTTATGTGCGAAGCGCTTTGAAGGAGGCTGACCTTGTCTTATGCGCTCTTGAAGCAAAAATGGAGCCTTACTTG 1280
 S Y V R T L L K E A D L V F L L P L E A K M E P Y L
 AAGCAGGCGTTTGTCTACAGCTATGTCTCAGCTGTTTCCGCTGATTGCGCTGAGCATCGTTCGATGCGGCTTTATTT 1360
 K Q A F V Y S Y V S Q L F P L I A L S I V A M P L Y F
 CGCAGTCACTCCGGGAGCTTCGCTCGTATCGTATGCGCGGCTTCTTGTCCAACGTGCTGCTGAAAGCGTGAATCAGG 1440
 A V T P G A S L V S Y A A V F V Q L L L L K A W N Q V
 TAATGGAAATGGCGAACGACTTCCAAAACGACCGCATGAAACCGATGGACGTCATCATTGCGTTTGGCGCGAATACA 1520
 M E W R T T F Q N D R S M K R M D V I I R F A A N T
 CTCGTTCTTTATTTGCTTTTCAATCTGTTATATGTATGCGCTTCTGCTATGTCATTATGGCTGTTCTTTATCTGTA 1600
 L V L Y F V F Q S V Y M Y A L L V Y V I M A V L Y L Y
 TATGCTTCCGCGAGCAAAACGGAACATTTAAATGGGAGGCAATATGAGTCCGAATGAGACGAAAGCAGCGTTTCT 1680
 M S S A A K R K T F K W E S H I E S E L R R K Q R F Y
 ATCGGATGGCAACCTGTTTACTGATGTGCGGCTTTGCGAAAGCAAGCCAAACGAGAGCTTATCTCGACTTTTGTCT 1760
 R I A N L F T D V P H L R K Q A K R R A Y L D F L L
 CGGCTTGGCGGTTTGAACAGCGCAAAACATTTGCCTATATGCTACCCGCGCTTTTGGCTTCGAGCGATTATTTGG 1840
 R L V P F E Q R K T F A Y M F T R A F L R S S D Y L G
 CATTCTTGTGCGAATACGATCGTTTCCGCGTATGATGATGCTCAGCAGCGCTGATTTGCGCGGTAATTTGA 1920
 I L V R L T I V F A L I I M Y V S A S P L I A A V L T
 CTGTTTGGCATTTCATACGGGCAITTCAGCTTCTGCGCTCTTCCGCTCACTTTGACCATCTGGCGCTTCAAGGCTG 2000
 V F A I F I T G I Q L L P L F G H F D H I A L Q E L
 TACCTGTGCAAAAGAAACAAGCTGAAAGCTATTTCTCTCTATTAAAACTGCATTTAGTATTCAAGCGCTGCTGAT 2080
 Y P V Q K E T K L K S Y F S L K T A L S I Q A L L M
 GTCTGTTGATCTGCTATGACGCGGCTTGACCGGTTTCTGTACGCGCTGATCGGTTCTGCGCTCTGATTTTGTG 2160
 S V A S A Y A A G L T G F L Y A L I G S A V L I F V V
 TTTTGGCTGCTATATGACGACCGGCTGAAAAGCAGGAAAGCTGGAACCTGAAAAGAGGTAATCAAATGACAGATA 2240
 L P A Y M T T R L K K H G K L -
 ecsC-> M T D N
 TCAGCTGCTGATGCAAGCCTTTGAATGGAAGGATTTTGGCAAGGATTCATGTTGAACGCTTTTTCGAAGC 2320
 Q L L M Q C E A L E W K M H F L R K D S M F E R F S K R
 GTGTCAGACGAGGTTGAATGAACGATTCCTGAAAAATCCATACGCTGCTACCGAGAGTGTGAAAAAAATGGTAGAA 2400
 V Q T K V N E R I P E K I H T V V T E S V K K M V E
 GCGACAATGGCGGCTCTAATATCATCACCTATAAAAGGATACAAGTGCAGTGTGCTGCTGAGTGAAGAAAAACGAATTGGC 2480
 A T M A G S N I I T Y K K D T S A L S L S E K N E L A
 GAAAAAACGATTGTTTCTATCAAAAAGTGGCAGCTGCTGAGGGAGTCGGCACCGGAGCGGGCGGCAITTTTAAAGCA 2560
 K K T I V S Y Q K V A A E G V G T G A G G I F L S I
 TTGCTGATTTTCCGCTGCTGCTTCAATTAAGATGAAGTGTATTTTACCTTGTCTTCCATTACGGCTTTGATGTCAG 2640
 A D F P L L L S I K M K C L F T L S S I Y G F D V K
 GATGCAAGAAGGATATTTTGTGCTGCTGTTTTCAGCTTTCGCTTTTCCAGTGTATGCGCGTAAGTCGCTTTTTC 2720
 D A Q E R I F L L L V F Q L A F S S D D G R K S L F S
 TGTCTCGAAAAATGGGAGACGAGAAAAAGAGCATCGACTGGAGAGTGTTCAGCAGGAATATCGCGATTATATCGAG 2800
 V I E N W E T E K K S I D W R V F Q Q E Y R D Y I D V
 TCGTGAAGCTTTTTCAGCTTTTCCGGGCGTAGGAGCGGAGTCGGCGGAATTCGCAATATAAGCTGCTGCTGCTGAGCTT 2880
 V K L F Q L L P G V G A A V G G I A N Y K L L A Q L
 GGAGAAACCGCAAGACATGTTTTCATGCTGAGGATTAAGGAAACAGCCGAGAGATAAACACTCCGGCTGTTTTTTA 2960
 -----> <-----
 G E T A R H V F H L R I L K E T A G E -
 TAGCTGATGATAGGTGATCGCAGCGCGGCAAGACCTTGGCCGCTGTCAGCATGGCTTTTTCGTTGATATCAAAATTCG 3040
 GATGGTGGTGGGAATAGACTCGCTCTGGCTGTTGGGAGCGGCGCTGTAAGAAAAAGTGCCTTCACGTTTGTGAAG 3120
 TAATAAGCAAAATCCTCGCGGCCATTTGGGTTACCGTCAATGACCTGCTGAACGCCCTCGGTATCTTTCGCGGTGCT 3200
 CACCAAGTGGTTCGTTTCTGCA

Fig. 3. Nucleotide sequence of the *ecs* operon. Deduced amino acid sequences of the three ORFs of the *ecs* operon (*ecsA*, *ecsB* and *ecsC*) are shown below the nucleotide sequence. Putative promoter sequences are indicated as -10 and -35. RBSs preceding the start codon of each ORF are underlined. Arrows below the sequence mark the stem-loop structure of the transcription termination site. In the *ecsA26* mutant, the G₇₃₈ (bold letter) was replaced by A₇₃₈. This mutation changes Gly₁₆₄ (bold letter) of EcsA to Glu₁₆₄.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Characteristics	Reference/source
Strain		
<i>E. coli</i>		
TGI	<i>supE bsdΔ5 thi Δ(lac-proAB) F' [traD36 proAB⁺ lacI^q lacZΔM15]</i>	Sambrook <i>et al.</i> (1989)
<i>B. subtilis</i>		
IH6821	<i>ecsA26</i> (pKTH10) (re-isolation from culture stock)	Kontinen & Sarvas (1988)
IH6531	<i>glyB133 hisA1 trpC2</i> (pKTH10)	Kontinen & Sarvas (1988)
Plasmid		
pGEM4Z	Ap ^r , <i>lacZ</i>	Promega
pKTH10	Km ^r , pUB110-derivative carrying α -amylase gene of <i>B. amyloliquefaciens</i>	Palva (1982)
pKTH3208	<i>glyB133</i> ⁺ , pJH101 carrying a 2.3 kb <i>Bam</i> HI– <i>Hind</i> III fragment of pKTH3267	This study
pKTH3211	<i>glyB133</i> ⁺ , pGEM4Z carrying a 2.3 kb <i>Bam</i> HI– <i>Hind</i> III fragment of pKTH3267	This study
pKTH3232	pGEM4Z containing a 3.2 kb <i>Sac</i> I fragment of λ K10	This study
pKTH3234	<i>ecsA26</i> , pGEM4Z carrying a 2.3 kb <i>Sac</i> I fragment of λ K10	This study
pKTH3267	<i>glyB133</i> ⁺ and <i>ecsA26</i> ⁺ , pGEM4Z carrying a 4.5 kb <i>Sac</i> I fragment of λ K3*	This study
pKTH3270	<i>ecsA26</i> , pGEM4Z containing a 1.7 kb <i>Eco</i> RI– <i>Hind</i> III fragment of λ K10	This study

* The λ K3 was isolated from a wild-type chromosomal library (Kontinen *et al.*, 1991).

HISP_ECOLI	MSEN--KLNVIDLHKRY---GEHEVLKGVSLQANAGDVISIIGSSGSGKSTFLRCINFL
MALK_ECOLI	MASV--QLQ--NVTKAW---GEVVVSKDINLDIHEGEFVVFVFGPSGCGKSTLLRMIAGL
OPPD_BACSU	MIRVTRLLEVLDKLAISFKTYGGEVQAIRGVNFHLDKGETLAIVGESGSGKSVTSQAIMKL
ECSA	MS----LLSVKDLTGGY---TRNPVLKNVSFTLEPNQIVGLIGLNGAGKSTTIRHIGL
	* * *
HISP_ECOLI	EKPSEGSIVVNGQTINLVRDKDGQLKVADKNQLRLLRTRLTMVFQHF--NLWSHMTVLEN
MALK_ECOLI	ETITSGDLFIKEKRMN-----DTPPAERG-----VGMVFQSY--ALYPHLSVAEN
OPPD_BACSU	IPMPPGYF---KRGEILFEGKDLVPLSEKEMQNVRGKEIGMIFQDPMTSLNPTMKVGKQ
ECSA	MDPHKGSIELNGKT--FAEDPEG-----YRSQFTYIPETP--VLYEELTLMEH
	* *
HISP_ECOLI	VMEAPIQVLGLSKQEARERAVK--YLAKVGIDERAQGYVHLSSGGOQQRVSIARALAME
MALK_ECOLI	-MSFGLKPAGAKKEVINQR-VN--QVAEVLQLAHLDRPKALSGGQQRVAIGRTLVAE
OPPD_BACSU	ITEVLFKHEKISKEAAKKRAVELLELVGIPMEKRVNQFPHEFSGGMRQVRVVIAMALAAN
ECSA	-LELTAMAYGLSKTEMEKR-LP--PLLKEFRMEKRLKWPFAHESKGMKQKVMIMCAFLAE
	* *
HISP_ECOLI	PEVLLFDEPTSLDPELVGELLRLIMQQLAEE-GKTMVVVTHEMGFAHVVSTHVI FLHQGK
MALK_ECOLI	PSVFLLDPEPLSNLDAALRVQMRIEISRLHKRLGRMTIYVTHDQVEAMTLADKIVVLDAGR
OPPD_BACSU	PKLLIADEPTTALDVTIQAQILELMKDLQKKIDTSII FITHDLGVVANVADRVAVMYAGQ
ECSA	PALYITIDEPFLGLDPLAINALLERMNE-AKKGASVLMSTHILATAERYCDSFIILHNGE
	* *
HISP_ECOLI	IEEEGAPEQLFGNPQ-----SPRLQ-----
MALK_ECOLI	VAQVGKPLELYHYPADRFVAGFIGSPKMNFLPVKVTATAIDQVQVELPMPNRRQVWLPE
OPPD_BACSU	IVETGTVDEIFYDPRHPYTWGLLASMPGLESGEEELTAIPGTTPDLTNPPKGD-----
ECSA	VRARGTLESEL-----REQF---GMKDAALDDLYLELTGK-----
	* *

Fig. 4. Alignment of EcsA and ATP-binding components of three bacterial ABC transporters: HisP of *Escherichia coli* (Kraft & Leinwand, 1987), MalK of *E. coli* (Gilson *et al.*, 1982) and OppD of *B. subtilis* (Perego *et al.*, 1991, Rudner *et al.*, 1991). The alignment does not extend to the C-termini of the polypeptides. The two ATP-binding motifs (Walker *et al.*, 1982) are double-underlined and the glycine/glutamine-rich sequence (Ames *et al.*, 1992) is underlined. Identical and related amino acids are shown by asterisks and dots, respectively. The highly conserved aspartic acid (D) is in bold. The arrow points to the *ecsA26* mutation site.

The sequence comparison leaves little doubt about the nature of EcsA as an ATP-binding component. The point mutation, *ecsA26*, is also consistent with this conclusion. The mutation replaces Gly₁₆₄ with a charged residue (Glu) close to the C-terminus of the Walker II motif of the ATP-binding site (Fig. 4) and could thus affect ATP binding.

EcsB and EcsC

ecsB and *ecsC* are predicted to encode polypeptides of 409 and 237 amino acid residues, respectively. No proteins showing significant homology with these proteins were found in the Swiss-Prot database.

In contrast to the ATP-binding component, hydrophobic components of ABC transporters do not show extensive

amino acid sequence homology. However, structural similarities can be found (Kerppola & Ames, 1992; Higgins, 1992). Studies on the topology of hydrophobic components have revealed a pattern of several membrane-spanning peptides with intervening periplasmic and cytoplasmic loops. Hydropathy profiles suggest a similar topology for a large number of hydrophobic components of ABC transporters. The structural homology is most striking in the C-terminal parts of the proteins (Kerppola & Ames, 1992). Most of the transporters are predicted to contain six transmembrane segments per hydrophobic component (Higgins, 1992), but the minimum structure has been proposed to consist of five membrane-spanning helices (Kerppola & Ames, 1992). In the case of large proteins, additional membrane spanners and loops can be found in the N-terminal end of the protein.

Figure 5. Hydrophobicity plot of (a) EcsA, (b) EcsB and (c) EcsC according to Kyte & Doolittle (1982) with the window of 20 amino acid residues. Hydrophobic sequences point upwards.

Hydropathy analysis (Kyte & Doolittle, 1982) of EcsB showed six highly hydrophobic regions characteristic of membrane-spanning segments (Fig. 5b). According to the algorithm described in Eisenberg *et al.* (1984), the hydrophobic segments were predicted to contain one membrane-spanning helix each. A large hydrophilic and charged loop could be identified in the C-terminal part of EcsB. The predicted structural features of EcsB fit to those of a hydrophobic component of an ABC transporter.

The hydropathy analysis of the EcsC polypeptide (Fig. 5c) also showed the presence of hydrophobic segments, but none of the algorithms used predicted the presence of membrane-spanning helices. Furthermore, the overall structural pattern is very different from that of EcsB or that of the hydrophobic components of ABC transporters. Therefore, the structural predictions do not give an indication that EcsC is a second hydrophobic component of the putative ABC transporter. Clearly, EcsC neither contains a signal sequence nor the consensus cleavage site of a lipoprotein signal peptide (Hayashi & Wu, 1990). Thus, there is no indication of EcsC being a lipoprotein, such as the binding protein found in the importers of *B. subtilis* (Mathiopoulos *et al.*, 1991; Perego *et al.*, 1991; Rudner *et al.*, 1991; Woodson & Devine, 1994), importers of other Gram-positive organisms (Alloing *et al.*, 1990), or the accessory component found in the subtilin exporter of *B. subtilis* (Chung *et al.*, 1992; Klein *et al.*, 1992).

The properties deduced for the *ecsA* and *ecsB* gene products strongly suggest that they form an ABC transporter. The role of this transporter in the production of the secreted proteins, in sporulation and competence which are affected pleiotropically by *ecs* mutations, remains to be elucidated.

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