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# A New Periplasmic Protein of *Escherichia coli* Which Is Synthesized in Spheroplasts but Not in Intact Cells

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**The gene *spy* from *Escherichia coli* has been cloned and sequenced. It encodes a precursor of a so far unknown 139-residue, rather basic periplasmic protein. It was not detectable immunologically in intact cells but was produced abundantly in spheroplasts. It could be a stress protein specific for spheroplasting.**

The subject of this communication was found accidentally. We wished to study the location and regulation of synthesis of a major protein of the *Escherichia coli* outer membrane, OmpA, when produced in spheroplasts. Spheroplasts of strain UH300 (15), a derivative of MC4100 (3), were prepared according to the method of Osborn et al. (18) by treatment of exponentially growing cells with lysozyme plus EDTA (2).

Figure 1 shows the protein composition of supernatants of spheroplasts. Standard spheroplast preparations (fivefold concentrated with respect to a culture growing up to an optical density at 600 nm of 0.8) were diluted twofold with 0.25 M sucrose in L broth (16) and were incubated for 90 min at 37°C in the absence or presence of 0.5% glucose. Spheroplasts were removed by centrifugation, and proteins in the supernatants were precipitated with ice-cold trichloroacetic acid (final concentration, 5%), washed with acetone, and solubilized by being boiled for 5 min in sample buffer. Equivalent amounts of whole cells were also boiled this way; insoluble material was removed by centrifugation. Sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis was done with Laemmli-type gels (11). An 18-kDa polypeptide, not present in this supernatant of freshly prepared spheroplasts, appeared upon incubation of the spheroplasts for 90 min at 37°C and became quite abundant when glucose (0.5%) was present during this time. The protein was eluted electrophoretically from such gels and subjected to automated Edman degradation, which yielded the N-terminal sequence ADTTTAAPADAKPMMMHH. This sequence was not found in any database; hence, we were dealing with an unknown protein, which we designated Spy (spheroplast protein y). A rabbit was used for the preparation of an antiserum (10). A library of chromosomal DNA from *E. coli* in phage λgt11 (24) was obtained commercially (Clontech, Palo Alto, Calif.). Plaques of the recombinant phage on strain Y1090 (25) were transferred to nitrocellulose filters (5) and treated with the antiserum, followed by peroxidase-coupled anti-rabbit immunoglobulin G serum (8). Although the anti-Spy serum appeared to be monospecific, plaques were also subjected to hybridization with a 21-mer degenerate oligonucleotide prepared according to residues 10 to 16 of the N-terminal sequence presented above. Hybridization (6) was performed with an oligonucleotide labeled with digoxigenin-dUTP and the DNA Labeling and Detection Kit (nonradioactive; Boehringer Mannheim). Since, indeed, plaques existed which reacted only with the antiserum, phage was isolated from a plaque reacting with both probes. The phage carried an insert of 4 kb and, by using the oligonucleotide for Southern analysis (21), the *spy* gene was located on an ~2.2-kb *Bsi*WI fragment which was ligated into the high-copy-number phagemid pBC SK+ (Stratagene, Heidelberg, Germany), opened with *Kpn*I, resulting in pSH1. It was sequenced (4, 19) with an A.L.F. DNA sequencer (Pharmacia-LKB, Freiburg, Germany) with the appropriate primers and the Auto Read Sequencing Kit of the same company. The result is shown in Fig. 2. There is one complete open reading frame (ORF) containing the N-terminal sequence determined for the Spy protein (except that there are only two instead of three methionines); the *spy* gene codes for a preprotein with a typical signal peptide. The 139-residue mature protein is highly charged (36% charged residues) and rather basic (Table 1). A search in the EMBL and GenBank databases revealed that 200 bases at the 3′ end of the insert are completely identical to those of clone pYU14, which is located at min 39.2 of the *E. coli* chromosome (22). Hence, *spy* is

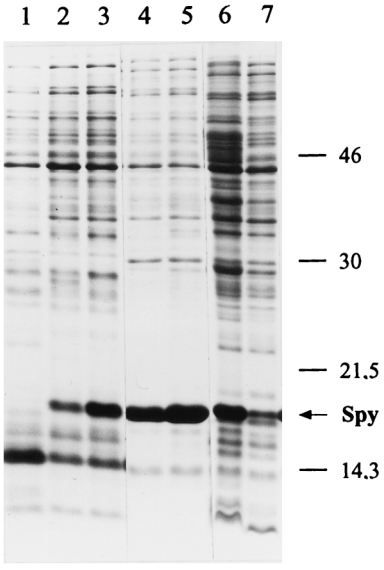


FIG. 1. Protein profiles. Lanes 1 to 5, polypeptides of supernatants of spheroplasts; 1, from freshly prepared spheroplasts; 2 and 3, upon incubation without or with glucose, respectively; 4 and 5, from spheroplasts carrying pSH2 incubated without or with glucose, respectively. Lanes 6 and 7 contain proteins of intact cells harboring pSH2 grown in the absence or presence of glucose, respectively. An electrophoretogram (12% gel) stained with Coomassie brilliant blue is shown. Numbers in the right margin show the positions of molecular weight marker proteins.

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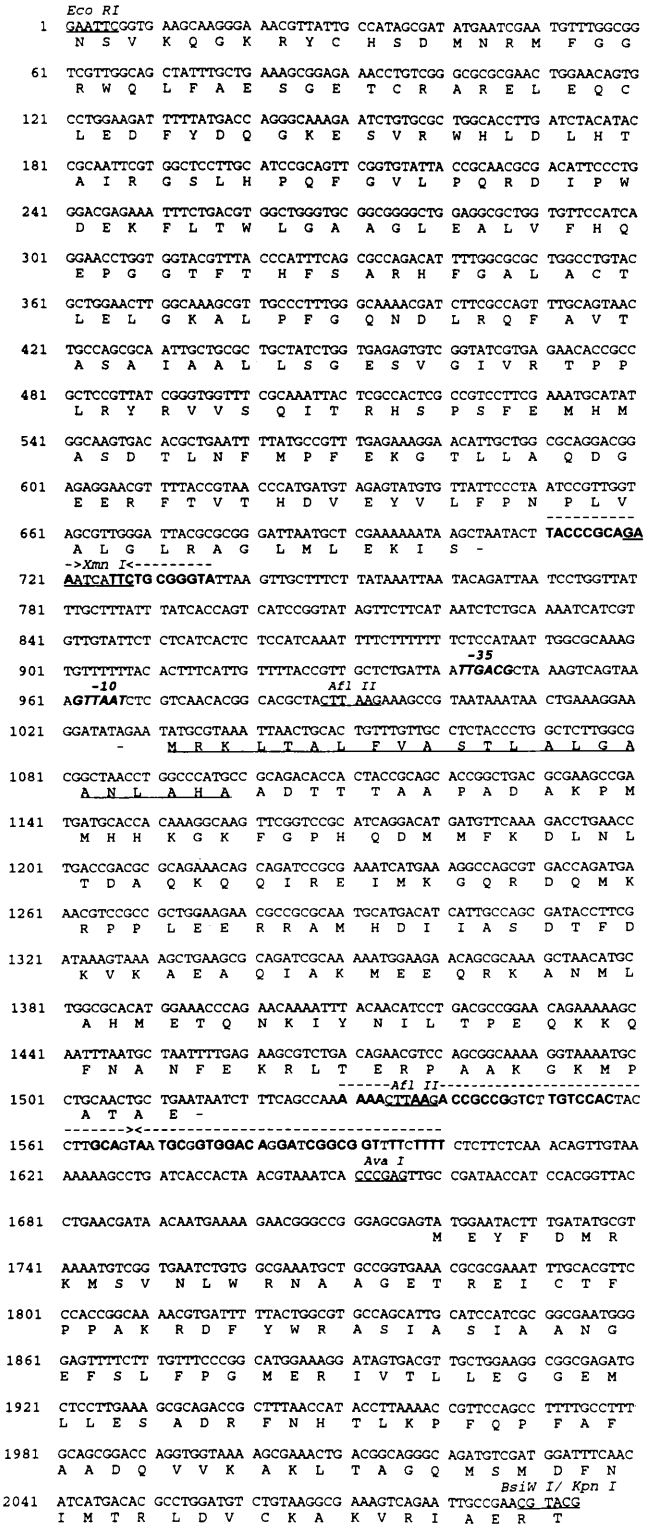


FIG. 2. DNA sequence of the *Bsi*WI fragment and deduced amino acid sequences. The signal peptide of Spy and the restriction sites used are underlined. The *Eco*RI site at position 1 is that of λgt11. The potential −35 and −10 regions are indicated (consensus sequences, 5′-TTG ACA and 5′-TAT AAT, respectively [9]); the dashed arrows delineate the candidates for palindromic transcription terminators (nucleotides potentially involved in base pairing within the terminators are shown in boldface type).

TABLE 1. Amino acid composition of Spy

| Residue | No. of mol/mol of Spy |
|---|---|
| Ala | 19 |
| Ile | 7 |
| Leu | 6 |
| Met | 11 |
| Phe | 5 |
| Pro | 8 |
| Trp | 0 |
| Val | 1 |
| Arg | 8 |
| His | 5 |
| Lys | 17 |
| Asn | 6 |
| Cys | 0 |
| Gln | 11 |
| Gly | 4 |
| Ser | 1 |
| Thr | 9 |
| Tyr | 1 |
| Asp | 9 |
| Glu | 11 |

located at this position. A potential gene fragment upstream from *spy* may code for 234 C-terminal residues; this sequence did not possess any significant identity with known polypeptides (SwissProt database). The gene fragment downstream from *spy* could code for 125 N-terminal residues of a protein. This sequence exhibits 39% identity in a 69-residue overlap with an ORF potentially coding for a 190-residue polypeptide of unknown function at the *hutC* region of *Pseudomonas putida* (1). Apparently, *spy* is not part of an operon encompassing one or the other of adjacent ORFs. The noncoding area upstream from *spy* possesses several characteristics of a promoter (Fig. 2). In addition, the ORF possesses a fairly strong ($\Delta G = -16$ kcal) palindromic transcriptional terminator. This most likely is also true for *spy*: between nucleotides 1537 and 1593, a GC-rich palindrome ($\Delta G = -34.4$ kcal) is followed by a poly(U) stretch typical for a *rho*-independent terminator. The orientation of *spy* in pSH1 is shown in Fig. 3. In order to avoid an influence of the *lac* promoter on the expression of *spy*, the gene was inverted. pSH1 was cleaved with *Xmn*I plus *Ava*I, and the 0.93-kb fragment was made blunt ended with the Klenow fragment of DNA polymerase I. It was then ligated into pBC SK+ opened with *Sma*I; the orientation of the insert in pSH2 (Fig. 3) was identified by restriction analyses. It was used for most further experiments.

Spy is a secreted protein, and this secretion is dependent on the Sec machinery (20); the presence of 10 mM sodium azide (17) in the medium of spheroplasts completely blocked secretion of Spy and caused accumulation of its precursor (data not shown). With Western blots (23) of electrophoretograms (SDS-polyacrylamide gels) of whole cells, the protein could not be detected, and it could also not be detected in cells lysogenic for the relevant recombinant λgt11, irrespective of whether glucose was present in growth medium. The protein was also not produced in cells incubated in the presence of 0.5 M sucrose or 0.35 M NaCl. Mutants have been described which are leaky for periplasmic proteins; among these are *tolB236*, *tolA1*, and *pal852* (11a, 12). These strains also did not produce Spy, not even when they carried *spy* on the low-copy-number plasmid pCL1920 (about five copies per cell [13]). Hence, neither osmotic stress nor a defective outer membrane caused expression of *spy*. However, in cells carrying the multicopy

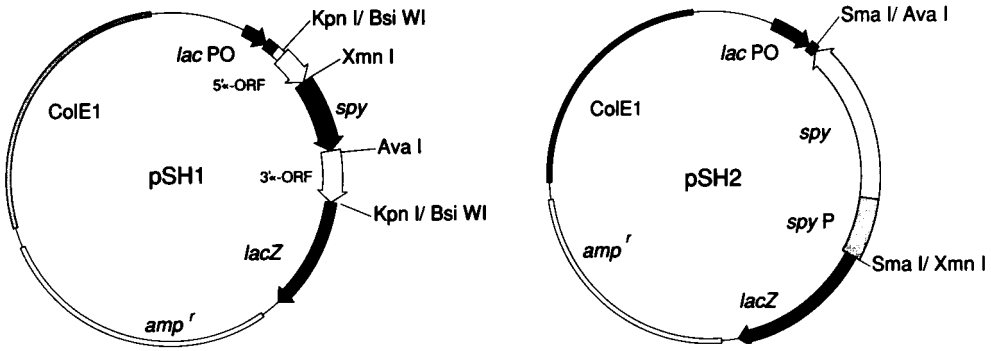


FIG. 3. Structures of pSH1 and -2. The cloned DNA in pSH1 is that shown in Fig. 2. In pSH2, *spy* P indicates the area between *Xmn*I and the ATG start codon for Spy synthesis (Fig. 2).

plasmid pSH2 (Fig. 3), Spy was easily detectable (Fig. 1) and could be identified by immunoelectron microscopy (Fig. 4). The polypeptide was located exclusively in the periplasmic space, an association with the plasma or outer membrane was not observed, and inclusion bodies were not apparent. We conclude that Spy was localized to the periplasmic space. The regulation of synthesis of Spy appears to be multifaceted. It was induced by spheroplasting, and this production was further increased in the presence of glucose (described above). A similar effect was found in spheroplasts carrying pSH2; however, exactly the opposite occurred in whole cells harboring this plasmid (Fig. 1). In trying to determine the function(s) of Spy, a *spy* null mutant was constructed. A deletion of *spy* was introduced into pSH1 by digestion with *Afl*II and religation (Fig. 2). The DNA with the deleted *spy* gene was cloned as a *Hin*dIII-*Bam*HI fragment (restriction sites in the vector) into plasmid pMAK705 (7), which was transformed into strain MC4100. The deletion was transferred to the chromosome by the method developed by Hamilton et al. (7). In brief, pMAK705 has a temperature-sensitive replicon and specifies resistance to chloramphenicol. Selection for this resistance at 44°C yields chromosomal cointegrates which grow very poorly at 28°C, and thus growth of these at 28°C in the presence of the antibiotic selects for resolution with either the wild-type or the deleted gene on the plasmid. This protocol resulted in both types of plasmids. A strain carrying the wild-type-size plasmid was cured by growth at 44°C in the absence of chloramphenicol. Chromosomal DNA of this strain was digested with various restriction enzymes. Electrophoretograms of the fragments were probed with the *Afl*II fragment (Fig. 2), labeled with digoxigenin-dUTP (described above). In contrast to DNA from strain UH300, there was no signal, and spheroplasts of the mutant did not produce Spy. Hence, the Δ*spy* mutant was viable. It had no obvious phenotype. The cells exhibited normal morphology and did grow in minimal medium (M9 [16]) with glucose, glycerol, succinate, acetate, or Casamino Acids as carbon sources at 28 or 44°C. There was no indication for a defect in the outer membrane. The mutant and the parental MC4100 did not differ in resistance or sensitivity to SDS, Zwittergent (Calbiochem), or dodecyltrimethylammonium bromide (the detergents were applied as 0.25 M aqueous solutions to filter paper on petri dishes). Spheroplasting may represent a new stress situation for *E. coli*, and induction of Spy synthesis could be part of a relevant response. It is anticipated that a detailed study of the function(s) of the *spy* promoter region will help reveal the physiological role of Spy.

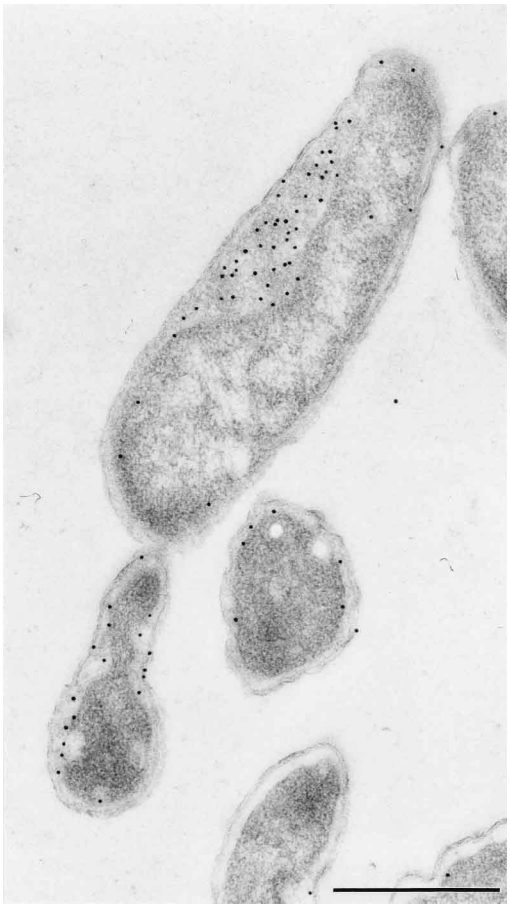

FIG. 4. Location of Spy in plasmolyzed cells carrying pSH2. Cells were grown in the absence of glucose (Fig. 1, lane 6) and processed for immunoelectron microscopy as described previously (14). Lowicryl HM20 sections were treated with Spy antiserum and labeled with 15-nm-diameter gold particles bound to protein A. Bar, 0.5 μm.

**Nucleotide sequence accession number.** The *Bsi*WI fragment sequence data appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number Y07714.

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