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Multicopy Suppression: an Approach to Understanding Intracellular Functioning of the Protein Export System

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***Escherichia coli* genes were cloned onto a multicopy plasmid and selected by the ability to restore growth and protein export defects caused by a temperature-sensitive *secY* or *secA* mutation. When *secA51* was used as the primary mutation, only clones carrying *groE*, which specifies the chaperonin class of heat shock protein, were obtained. Selection using *secY24* yielded three major classes of genes. The first class encodes another heat shock protein, HtpG; the most frequently obtained second class encodes a neutral histonelike protein, H-NS; and the third class, *msyB*, encodes a 124-residue protein of which 38 residues are acidic amino acids. Possible mechanisms of suppression as well as the significance and limitations of the multicopy suppression approach are discussed.**

Protein translocation across the *Escherichia coli* cytoplasmic membrane is facilitated by proteinaceous factors. Recent progress in the biochemical dissection of the process has provided the following outlines. Secretory (periplasmic and outer membrane) proteins are initially synthesized as precursor molecules with N-terminal extensions, the leader or signal sequences. The SecB protein in the cytoplasm associates with many (although not all) secretory protein precursors and maintains them in translocation-competent conformations by preventing premature folding (21). The precursors are then targeted to the cytoplasmic membrane, probably by combined action of SecB and SecA (12, 42), a peripheral membrane component (31) with an ATPase activity (25). In addition to SecA, the proton motive force across the membrane (36) and the integral membrane components SecE and SecY are essential for subsequent movement of the polypeptide across the membrane (1, 4).

The Sec proteins mentioned above had all been implicated in protein translocation by genetic studies (for a review, see reference 35). From a genetic point of view, it is important to ask to what extent the in vitro reaction mimics the in vivo processes. For instance, the roles of SecD and SecF, additional integral membrane components that have been assigned genetically (9), remain to be shown in vitro. Also, it is not known whether *E. coli* possesses additional genes that specify factors involved in protein export. As an approach to identifying novel export-related genes, we searched for genes that circumvented the export-defective phenotypes of *sec* mutations when cloned onto a multicopy plasmid. The rationale behind this approach was that an increased abundance of some cellular component may either help the functioning of the mutated gene product (if they act in concert) or compensate for the retardation caused by the mutated gene product (if they act in sequence). Suppression due to an increased dose may also be expected for genes that control expression of export-related genes or that affect protein folding and assembly (44). We reasoned that even the latter groups of genes will be of potential interest, since they may provide some useful information about the intracellular functioning of the protein export system.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used were all *E. coli* K-12 strains and are listed in Table 1.

Plasmids. Manipulations of DNA in vitro were done essentially as described by Sambrook et al. (33). Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, exonuclease III, and other DNA-modifying enzymes were purchased form Takara Shuzo or Toyobo. Plasmid pNO1575 (18), a pBR322 derivative carrying the *lac* promoter and the multicloning site from pUC9, was used as a cloning vector. pNO1575H (17), a derivative of pNO1575 with a frameshift mutation in the *Hin*dIII site, was used as a control plasmid without a chromosomal insert; the mutation reduces the *lac* promoter-dependent elevation of the *bla* (β-lactamase) expression that may affect protein export in *secY24* mutant cells (17).

An *E. coli* genomic library was the starting material for the selection of multicopy suppressors and was constructed by partially digesting chromosomal DNA of strain W3110 with *Sau*3AI, recovering 5- to 15-kb fragments after electrophoresis through a 1% low-melting-point agarose gel (Bethesda Research Laboratories), ligating the fragments with *Bam*HI-digested pNO1575, transforming strain KI262, and finally extracting plasmid DNAs from pooled transformants. Plasmids selected by suppression activity were given names starting with pMSY (multicopy suppressor of *secY24*). pKY184 was constructed by ligating the 980-bp *Hin*dIII-*Aat*II fragment of pUC119 (45) with the 3,868-bp *Hin*dIII-*Aat*II fragment of pNO1575.

Plasmid pMSY3 carried the *htpG-adk* region. Its 3.3-kb *Eco*RI fragment was cloned into the *Eco*RI site of pNO1575, yielding pMSY301.

Plasmid pMSY5, pMSY6, and pMSY12 (*msyA* class) carried DNA from the 27.5-min region of the *E. coli* chromosome. pMSY12 was digested with *Eco*RI and self-ligated to yield a plasmid named pMSY1201, which was then treated with *Hin*dIII and *Aat*I, blunted, and ligated to yield pMSY1205. Similar treatment of pMSY1201 with *Hin*dIII and *Hpa*I yielded pMSY1210. A 2.9-kb *Sal*I fragment of

TABLE 1. *E. coli* strains used in this study

Strain	Characteristic(s)	Reference
MC4100	Δ(*argF-lac*)*U169 araD rpsL relA flbB deoC ptsF rbsR*	5
W3110	A prototrophic strain	Our stock
MM52	MC4100 *secA51*(Ts)	30
IQ85	MC4100 *secY24*(Ts) *zhd-33*::Tn*10 rpsE*	40
KI330	MC4100 *secY100*(Ts) *zhd-33*::Tn*10 rpsE*	17
KI200	MC4100 *rplO215*(Am) *zhd-33*::Tn*10-rpsL*$^+$ (φ80 *sus2* p*supFts6*)	16
CK1953	MC4100 *secB*::Tn*5*	22
MM18	MC4100 [λ(*malE-lacZ*)(Hyb)72-47]	2
KI262	Δ(*pro-lac*) *thi*/F′ *lacI*^{q} ZM15 *Y*$^+$ *pro*$^+$	Our stock

pMSY6 was cloned into the *Sal*I site of pNO1575 to yield pMSY606 (see Fig. 3).

Plasmid pMSY7 (*msyB*) carried an approximately 3.3-kb fragment derived from the 23-min region. pMSY7 was digested with *Eco*RI and ligated to yield pMSY701 (see Fig. 6). pMSY701 was digested with *Bst*EII and *Eco*RI, blunted, and ligated to yield pMSY702; digested with *Xho*I and *Eco*RI, blunted, and ligated to yield pMSY703; digested with *Hin*dIII and *Xho*I, blunted, and ligated to yield pMSY704; and digested with *Aat*I and *Eco*RI, blunted, and ligated to yield pMSY705. A 455-bp *Bbi*II fragment of pMSY701 was blunted, and digested with *Xho*I, and the resulting 365-bp fragment was ligated with pMSY701 that had been digested with *Xho*I and *Bst*EII and blunted, yielding pMSY706. This 365-bp fragment was ligated with *Xho*I-*Eco*RI-digested and blunted pMSY701 to yield pMSY707. pMSY701 was digested with *Xho*I, blunted, and ligated to yield pMSY708.

Construction of additional subclones and sequencing. DNA fragments to be sequenced were cloned onto pKY184. pKY185 and pKY186 carried the *msyA* region; the fragment used for construction of pMSY606 was cloned in both orientations. pMSY511 through pMSY524 contained a series of internal deletions in pKY185 that had been introduced by treating pKY185 successively with *Bam*HI plus *Kpn*I, exonuclease III, mung bean nuclease, and T4 DNA ligase. pMSY531 through pMSY542 were similarly constructed with pKY186.

pKY187 and pKY188 contained the *msyB* region; a 2.7-kb *Hin*dIII (vector part)-*Eco*RI fragment of pMSY7 was blunted and cloned in both orientations. A series of deletions were introduced into pKY187 and pKY188 by either *Bst*EII-*Eco*RI or *Xho*I-*Eco*RI double digestion followed by blunt-end formation and ligation.

Single-stranded DNAs were prepared from the plasmids described above by packaging into M13 phage (45), and nucleotide sequences were determined by the chain termination method (34) using Sequenase version II (United States Biochemicals). Both strands were determined for the entire sequenced regions; synthetic oligonucleotide primers were used as required.

Media, growth, pulse-labeling, immunoprecipitation, and gel electrophoresis. The agar media used were L (27), peptone (18), and M9-glucose (27) agar media. Ampicillin (50 μg/ml) was added to select and maintain plasmid-bearing cells. For protein pulse-labeling, cells were grown in minimal M9 medium containing 0.4% glucose or 0.4% maltose, 20 μg of each amino acid except methionine and cysteine per ml, and 50 μg of ampicillin per ml. Pulse-labeling was initiated by adding [^{35}S]methionine (1,000 Ci/mmol; American Radiolabeled Chemicals) at about 50 μCi/ml and terminated by addition of an equal volume of 10% trichloroacetic acid. Chase with unlabeled methionine was initiated by adding 200 μg of L-methionine per ml. The trichloroacetic acid-denatured proteins were solubilized in sodium dodecyl sulfate (SDS), diluted, immunoprecipitated with antiserum against the OmpA protein or the maltose-binding protein, and subjected to gel electrophoresis, autoradiography, and densitometric quantitation of the precursor and mature forms, essentially as described previously (43).

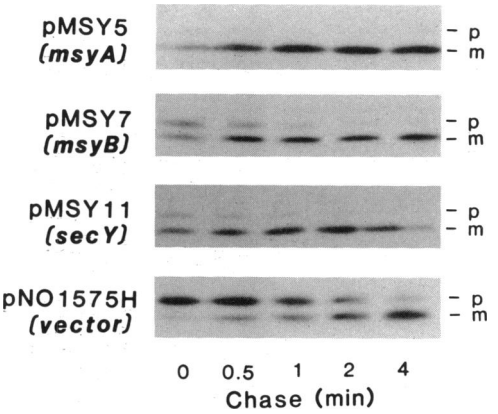

FIG. 1. Processing kinetics of OmpA in plasmid-bearing cells. IQ85 (*secY24*) cells carrying pMSY5 (*msyA*), pMSY7 (*msyB*), pMSY11 (*secY*), or pNO1575H (no chromosomal insert) were grown at 30°C, shifted to 42°C for 2 h, pulse-labeled with [^{35}S]methionine for 0.5 min, and chased with unlabeled methionine for the indicated periods. OmpA immunoprecipitates were electrophoresed and autoradiographed. p, precursor; m, mature.

Nucleotide sequence accession numbers. The nucleotide sequence in Fig. 5 has been deposited in the EMBL data library under accession number X59939. The sequence (not shown) of the 1,993-bp *Sal*I fragment described in Results has been deposited in the EMBL data library under accession number X59940.

RESULTS

Multicopy suppressors of *secY24*. A mixture of recombinant plasmids randomly carrying chromosomal fragments was introduced into strain IQ85, which carries the *secY24* mutation (40). Temperature-resistant transformants were selected on minimal agar plates at 42°C, and plasmid DNAs were extracted and reintroduced into IQ85 to confirm the suppression activity. We thus obtained 25 clones from samples that would have formed about 120,000 transformants. Restriction patterns of the chromosomal inserts in these plasmids indicated that at least 18 clones were derived from independent origins. They were compared with the physical map of the whole *E. coli* genome (20). We were able to provisionally assign locations of 12 independent clones to several loci. One of them (pMSY11) included *secY* itself and should have been selected by true complementation. The remaining 11 clones fell into six groups with approximate map positions of 11, 23, 26, 27.5, 55, and 97 min. The 27.5-min class consisted of six clones (pMSY5, pMSY6, pMSY8, pMSY12, pMSY22, and pMSY24) and others) with partially overlapping restriction patterns. The 11-min class (pMSY3) appeared to contain *htpG*, which encodes a heat shock protein (2).

We then examined whether these clones indeed improved protein export that had been retarded by the *secY24* muta-

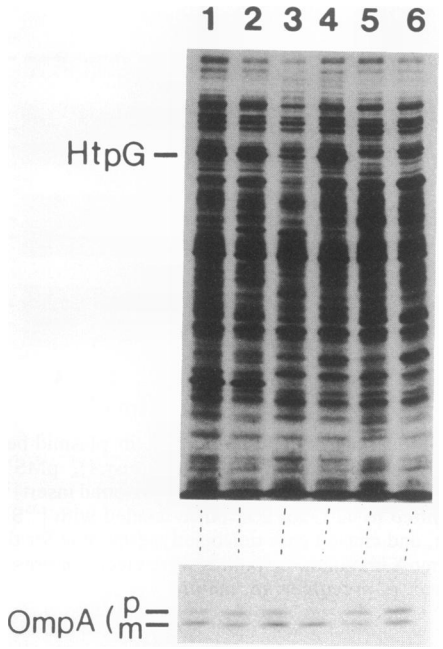

FIG. 2. HtpG overproduction and OmpA export in plasmid-bearing *secY24* cells. IQ85 (*secY24*) cells harboring pMSY3 (lanes 1 and 2), pMSY301 (lanes 3 and 4), or pNO1575H (lanes 5 and 6) were grown on M9-glycerol (odd-numbered lanes) or M9-glucose (even-numbered lanes) medium at 30°C, shifted to 42°C for 2 h, and pulse-labeled with [^{35}S]methionine for 2 min. The upper panel shows patterns of total labeled proteins, whereas the lower panel shows OmpA immunoprecipitates.

tion. Pulse-labeling experiments showed a marked improvement in export (decrease in the precursor-to-mature protein ratio of pulse-labeled OmpA) for cells carrying the 23-min plasmid (pMSY7) or a 27.5-min class of plasmids (data not shown). Some other plasmids, including pMSY3 (the *htpG* class), showed significant but weak effects (see Fig. 2). In the experiment whose results are shown in Fig. 1, IQ85 cells harboring a plasmid were exposed to 42°C for 2 h, pulse-labeled with [^{35}S]methionine for 30 s, and chased with unlabeled methionine. OmpA immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Plasmids pMSY5 (a 27.5-min clone) and pMSY7 (the 23-min clone) markedly accelerated the processing rate of OmpA. We named the responsible genes on pMSY5 and pMSY7 *msyA* and *msyB*, respectively. We decided to subject these two classes, which have clear export-enhancing activities, as well as the 11-min class containing *htpG*, to further studies.

Specificity of suppression. Neither of the plasmids pMSY5 (*msyA*) and pMSY7 (*msyB*) improved the growth of strain KI200, which carries *rplO215*, an amber mutation that reduces the expression level of *secY* because of a polarity effect (16). pMSY5 but not pMSY7 improved growth and protein export of strain KI330, which carries another temperature-sensitive (Ts) mutation, *secY100* (17). Neither *msy* plasmid affected the growth or export phenotypes of *secA51*(Ts) (30) and *secB*::Tn*5* (22) mutations. They also did not affect the maltose-sensitive phenotype of strain MM18, which carries the export-blocking *malE-lacZ* gene fusion (3). Plasmid pMSY3 (carrying *htpG*) suppressed *secY24* and *secY100* but not the other *sec* mutations. In summary, the multicopy suppressors obtained suppressed only the *secY* mutations; the *msyB* clone was specific for *secY24*.

Multicopy suppressors of *secA51*. We repeated a similar selection by using strain MM52, which carries the *secA51*(Ts) mutation. Of 13 independent plasmid clones obtained, 7 were shown by restriction analysis to contain *secA* itself, whereas 6 clones were shown to carry the *groEL* and *groES* genes. Van Dyk et al. (44) reported that overproduction of the GroE heat shock proteins suppressed several unrelated temperature-sensitive mutations, including *secA51* and *secY24*. However, our selection using *secY24* did not yield a *groE* clone. Consistent with this result, the *groE* clones isolated as described above did not suppress the

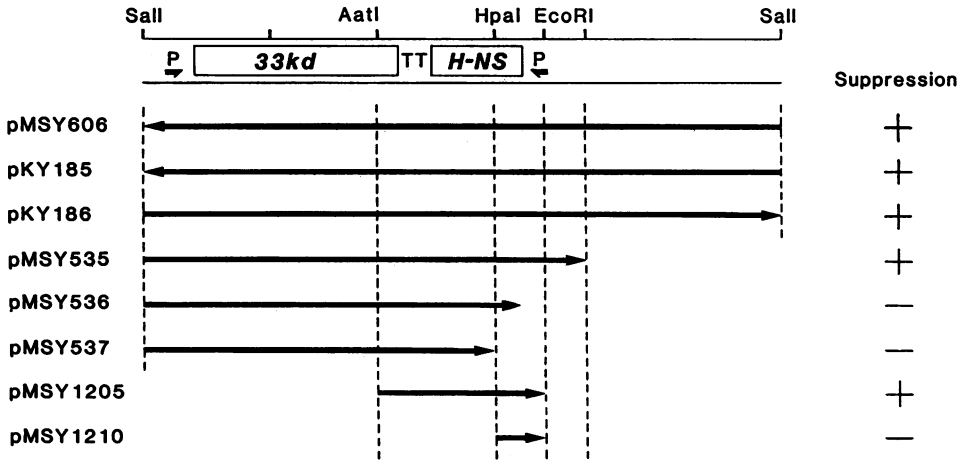

FIG. 3. Structure of the *msyA* region and suppression activities of plasmid derivatives. Some restriction enzyme cleavage sites and the two ORFs (33kd and H-NS) revealed by sequencing are shown at the top. P and T represent the putative promoter and terminator of transcription, respectively. The 33-kDa ORF is oriented clockwise, whereas the H-NS ORF is oriented counterclockwise, on the chromosome map. The sequence of the H-NS region contained the following differences, in addition to those noted by May et al. (26), from the sequence reported by Pon et al. (32): A at nucleotide 601 (numbering according to reference 32) was missing, and G was inserted between nucleotides 1315 and 1316. The chromosomal region carried in each plasmid is shown by an arrow which indicates the direction of transcription directed by the *lac* promoter on the vector. + or − at the right indicates the presence or absence of suppression activity, respectively.

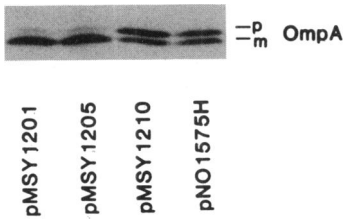

FIG. 4. Enhancement of OmpA export by plasmids carrying the H-NS ORF. IQ85 cells carrying a plasmid as indicated were shifted to 42°C for 2 h, pulse-labeled with [^{35}S]methionine for 2 min, and then subjected to immunoprecipitation and gel electrophoresis. p, precursor; m, mature.

secY24 mutation. Also, several GroE-overproducing plasmids isolated by ourselves or others exhibited negligible or very small effects on the temperature sensitivity of the *secY24* mutant. GroE in cells carrying one of these plasmids, pKY206, amounted to as much as about 40% of the total protein (15).

Overproduction of HtpG in the *secY24* mutant improves protein export. We constructed a plasmid (pMSY301) carrying only *htpG* as an intact chromosomal gene oriented in the same transcriptional direction as the *lac* promoter on the vector. pMSY301 improved the growth of IQ85 on minimal glucose agar at 42°C, but it inhibited the growth of even *sec*$^+$ cells when minimal glycerol medium was used (note that *lacI* was deleted from the strains used). Electrophoretic patterns of the whole-cell proteins shown in Fig. 2 indicated that cells harboring pMSY301 overproduced HtpG at a higher level in the glycerol medium (lane 4) than in the glucose medium (lane 3), suggesting that *htpG* on pMSY301 was dually promoted by its own promoter and the *lac* promoter. Processing (export) of OmpA was accelerated markedly by pMSY301 in the glycerol medium (Fig. 2, lane 4). pMSY3 (the original clone) also overproduced HtpG and slightly improved OmpA processing (lanes 1 and 2). Thus, increased expression of HtpG can lead to improved protein export in the *secY24* mutant cells.

Characterization of *msyA*. Subcloning of DNA fragments derived from the *msyA* class plasmids showed that an approximately 2.0-kb *Sal*I fragment (Fig. 3) contained the suppression activity. This DNA region could not be cloned onto pUC119 (for DNA sequencing), and rare transformants always contained deletions in this region, suggesting that this fragment is incompatible with pUC119, whose copy number is higher than that of pNO1575. We therefore constructed a new vector (pKY184) for dideoxy sequencing, in which both the multicloning site and the M13 phage-packaging site were derived from pUC119 (45) while the remaining region was derived from pNO1575; the 1,993-bp *Sal*I fragment was cloned and sequenced (data not shown). Within this region, there were two open reading frames (ORFs) with different orientations, each of which was flanked by promoterlike and terminatorlike sequences (Fig. 3). One of the ORFs (the

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GATCATGATTACGGCCCGCGCTCAAGCGTTTTCGTCCCGTTGTTTGCCGTTGAGCAGGCT      60
 D  H  D  Y  G  P  R  S  S  V  F  V  P  L  F  A  V  E  Q  A
GCGACCACGACCGGAACCTGGATGCTGGCACGGATGTCCGGCGCATGTCTGGTGCCCTTC     120
 A  T  T  T  G  T  W  M  L  A  R  M  S  G  A  C  L  V  P  F
GTTCCACGCCGTAAGCCAGATGGCAAAGGGTATCAATTGATTATGCTGCCGCCAGAGTGT     180
 V  P  R  R  K  P  D  G  K  G  Y  Q  L  I  M  L  P  P  E  C
TCTCCGCCACTGGATGATGCCGAAACTACCGCCGCGTGGATGAACAAAGTGGTCGAAAAA     240
 S  P  P  L  D  D  A  E  T  T  A  A  W  M  N  K  V  V  E  K
TGCATCATGATGGCACCAGAGCAGTATATGTGGTTACACCGTCGCTTTAAAACACGCCCG     300
 C  I  M  M  A  P  E  Q  Y  M  W  L  H  R  R  F  K  T  R  P
GAAGGCGTTCCTTCACGCTATTAAATCTCCCATGCCGGATGCTTCAGAATGGCATCCGGC     360
 E  G  V  P  S  R  Y  *
ATTACCACAGCAAATCCCCCTGATTTAGCGATAAAAGCTCTCTGGATTGCGCCCCCTGGA     420
                                          -35
AGTCGGGCGCATAATTAGTGTGCTTATCTTTTCTTCTTATGTTCACCGCGCCTGGCGCAC     480
          -10
CAACAGCGGATTGCTATGTCACCCTGTGAAAATGACACCCCTATAAACTGGAAACGAAAC     540
     ORF1  M  S  P  C  E  N  D  T  P  I  N  W  K  R  N
CTGATCGTCGCCTGGCTAGGCTGTTTTCTTACCGGTGCCGCCTTCAGTCTGGTAATGCCC     600
 L  I  V  A  W  L  G  C  F  L  T  G  A  A  F  S  L  V  M  P
TTCTTACCCCTCTACGTTGAGCAGCTTGGCGTTACCGGTCACTCCGCCCTGAATATGTGG     660
 F  L  P  L  Y  V  E  Q  L  G  V  T  G  H  S  A  L  N  M  W
TCCGGTATTGTCTTCAGCATTACATTTTTATTTTCCGCCATCGCCTCACCGTTTTGGGGT     720
 S  G  I  V  F  S  I  T  F  L  F  S  A  I  A  S  P  F  W  G
GGACTCGCCGACCGTAAAGGCCGAAAACTCATGCTATTACGCTCTGCCCTCGGCATGGGC     780
 G  L  A  D  R  K  G  R  K  L  M  L  L  R  S  A  L  G  M  G
ATCGTGATGGTGTTGATGGGGCTGGCACAAAATATCTGGCAGTTTTTGATCCTGCGGGCG     840
 I  V  M  V  L  M  G  L  A  Q  N  I  W  Q  F  L  I  L  R  A
CTTCTTGGGTTACTTGGCGGATTTGTCCCCAACGCTAATGCTCTTATCGCCACACAAGTA     900
 L  L  G  L  L  G  G  F  V  P  N  A  N  A  L  I  A  T  Q  V
CCGCGTAATAAAAGCGGCTGGGCGCTGGGTACGCTCTCCACAGGCGGCGTTAGTGGTGCG     960
 P  R  N  K  S  G  W  A  L  G  T  L  S  T  G  G  V  S  G  A
TTGCTCGGCCCAATGGCTGGCGGCCTGCTCGCCGATAGCTACGGCTTACGTCCGGTATTC    1020
 L  L  G  P  M  A  G  G  L  L  A  D  S  Y  G  L  R  P  V  F
TTTATTACCGCCAGTGTGCTCATACTCTGCTTTTTCGTCACCCTGTTTTGCATCAGAGAA    1080
 F  I  T  A  S  V  L  I  L  C  F  F  V  T  L  F  C  I  R  E
AAATTCCAGCCGGTCAGCAAAAAAGAGATGCTGCACATGCGGGAAGTGGTGACATCACTT    1140
 K  F  Q  P  V  S  K  K  E  M  L  H  M  R  E  V  V  T  S  L
AAAAACCCGAAACTGGTACTCAGCCTGTTTGTCACTACGTTAATCATCCAGGTGGCGACG    1200
 K  N  P  K  L  V  L  S  L  F  V  T  T  L  I  I  Q  V  A  T
GGCTCAATTGCCCCCATTCTGACGCTGTATGTCCGCGAACTGGCGGGTAACGTCAGTAAC    1260
 G  S  I  A  P  I  L  T  L  Y  V  R  E  L  A  G  N  V  S  N
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GTCGCCTTTATCAGTGGCATGATCGCCTCGGTGCCAGGCGTGGCGGCTCTGCTAAGTGCA    1320
 V  A  F  I  S  G  M  I  A  S  V  P  G  V  A  A  L  L  S  A
CCACGACTCGGCAAACTTGGCGATCGAATCGGACCCGAAAAGATCCTGATTACAGCGCTG    1380
 P  R  L  G  K  L  G  D  R  I  G  P  E  K  I  L  I  T  A  L
ATCTTTTCTGTACTGCTGTTGATCCCAATGTCTTACGTTCAGACGCCATTGCAACTTGGG    1440
 I  F  S  V  L  L  L  I  P  M  S  Y  V  Q  T  P  L  Q  L  G
ATTTTACGTTTTTTGCTCGGTGCCGCCGATGGTGCACTACTCCCCGCCGTACAGACACTG    1500
 I  L  R  F  L  L  G  A  A  D  G  A  L  L  P  A  V  Q  T  L
TTGGTTTACAACTCGAGCAACCAGATCGCCGGGCGTATCTTCAGCTATAACCAATCGTTT    1560
 L  V  Y  N  S  S  N  Q  I  A  G  R  I  F  S  Y  N  Q  S  F
CGTGATATTGGCAACGTTACCGGACCATTGATGGGAGCAGCGATTTCAGCGAACTACGGT    1620
 R  D  I  G  N  V  T  G  P  L  M  G  A  A  I  S  A  N  Y  G
TTCAGAGCGGTATTTCTCGTCACCGCTGGCGTAGTGTTATTCAACGCAGTCTATTCATGG    1680
 F  R  A  V  F  L  V  T  A  G  V  V  L  F  N  A  V  Y  S  W
AACAGTCTACGTCGTCGTCGAATACCCCAGGTATCGAACTGATTTTTCGCCTTTCATACT    1740
 N  S  L  R  R  R  R  I  P  Q  V  S  N
TGCAAAAGCGGAGAATCAGCTATCCTTTTCCCTGAAACCTCATCAACTCAAAGGGAGAAT    1800
                                                    SD
CGTGATGACCATGTACGCAACGCTTGAAGAAGCCATTGACGCTGCACGCGAAGAATTTCT    1860
ORF2  M  T  M  Y  A  T  L  E  E  A  I  D  A  A  R  E  E  F  L
TGCAGACAACCCCGGCATCGACGCCGAAGATGCGAATGTGCAACAGTTCAATGCCCAAAA    1920
 A  D  N  P  G  I  D  A  E  D  A  N  V  Q  Q  F  N  A  Q  K
ATACGTTTTGCAGGACGGCGACATCATGTGGCAAGTTGAGTTTTTTGCCGACGAAGGGGA    1980
 Y  V  L  Q  D  G  D  I  M  W  Q  V  E  F  F  A  D  E  G  E
AGAAGGTGAATGTTTACCTATGCTTAGCGGTGAAGCCGCGCAAAGTGTTTTTGATGGCGA    2040
 E  G  E  C  L  P  M  L  S  G  E  A  A  Q  S  V  F  D  G  D
CTATGATGAGATAGAGATACGCCAGGAGTGGCAGGAAGAGAATACATTACATGAATGGGA    2100
 Y  D  E  I  E  I  R  Q  E  W  Q  E  E  N  T  L  H  E  W  D
CGAGGGGGAATTTCAGCTTGAGCCACCGCTGGATACCGAGGAAGGACGCGCAGCAGCTGA    2160
 E  G  E  F  Q  L  E  P  P  L  D  T  E  E  G  R  A  A  A  D
TGAGTGGGATGAACGTTAATCACTCATACGGGCCATGATGAATGTCGATCGGCAGCAGTA    2220
 E  W  D  E  R
AAGTATCAAAAACCAGAGAGAATGGCAGATCAAGGATCGTGACATAACGCCAGGCGGAGT    2280
CACGCACATCCCATTGCACACCGGGATAATATTGGTTGCCATGCCCCTGCCCCGGTATAG    2340
TGCGACTAATAATGCTGCCACAGCCACTCAGCAAGGTCACCATGATGCTCACCACAATTA    2400
                                  (BstEII)
AACGCATTTGATACCTCTTTCGTAAAAAAATACCGGCATAACGCCGGTATTTTTATTGGC    2460
TCGTTGTTTTTGCCGGATGCGGCGTGAACGCCTTATCCGACCCACAAAGTCTTGCAAAAT    2520
CAATAAATTGCAGGAACGATGTAGGCCTGATAAGCGTAGCGCATCAGGCAACTACGTTTT    2580
TATTGCGAAGCCGCATCCGGTTTACGCAATGCCAGTGGATTGAGCTTTATCCCTTCGTAA    2640
TAACTCACCCATGAAGAATATCTCTCCGGGGAATTC                            2676
                                  (EcoRI)
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FIG. 5. Nucleotide and derived amino acid sequences of the *msyB* region of the chromosome. The putative promoter sequences for the *msyB* operon are marked by −35 and −10, whereas terminator structure is indicated by half-arrows showing a dyad symmetry. SD, Shine-Dalgarno sequence.

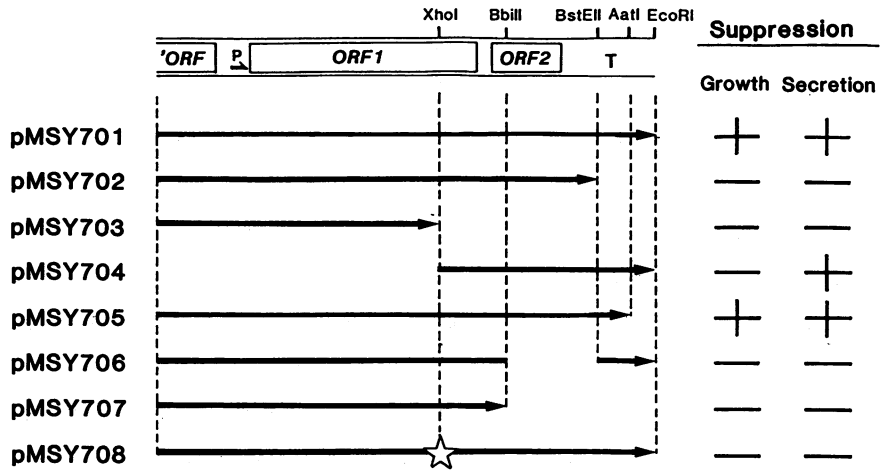

FIG. 6. Structure of the *msyB* region and suppression activities of plasmid derivatives. Some restriction enzyme cleavage sites as well as the ORFs revealed by sequencing are indicated at the top. P and T represent the putative promoter and terminator of transcription, respectively. The transcriptional orientation is counterclockwise on the standard chromosomal map. The chromosomal region carried in each plasmid is shown by arrows that indicate the direction of transcription directed by the *lac* promoter on the vector. pMSY706 has an internal deletion. The star in pMSY708 indicates a frameshift mutation. Suppression activities with respect to cell growth (Growth) and OmpA export (secretion) are shown on the right.

33-kDa ORF) was oriented clockwise on the chromosome and contained 302 codons with an ATP-binding motif in the C-terminal region; no homologous sequence was found in the EMBL data base. The other ORF consisted of 137 codons whose alignment perfectly matched the sequence of a neutral histonelike protein, H-NS (8). Pon et al. (32) reported the DNA sequence of *hns* (note, however, that the chromosomal map position assigned by Pon et al. [32] was incorrect; see the reports of May et al. [26] and Higgins et al. [13]). The DNA sequence we determined contained several base differences (see legend to Fig. 3) from the one reported by Pon et al. (32). Göransson et al. (11) as well as May et al. (26) also reported that they sequenced *hns*.

Plasmid pMSY1205, which carries only the H-NS ORF as an intact chromosomal gene, was active in suppression (Fig. 4). The suppression activity was lost when a carboxy-terminal region of H-NS was deleted (pMSY1210). Of the plasmids used for sequencing, pMSY535 was active, but pMSY536 (lacking the promoter region) and pMSY537 (lacking an amino-terminal region) were inactive while they contained the intact 33-kDa ORF (Fig. 3). We conclude that the gene defined by *msyA* is identical to the structural gene for H-NS.

Characterization of *msyB*. The map position (23 min) assigned for *msyB* was confirmed by hybridizing a ^{32}P-labeled *Xho*I-*Bst*EII fragment of pMSY7 with a filter-immobilized genomic DNA library (20); hybridization was observed specifically with clones 1H7 and E4H10S. Of the approximately 3.3-kb chromosomal insert in pMSY7, a 2.7-kb *Sau*3AI-*Eco*RI fragment retained the suppression determinant (pMSY701 in Fig. 6). We cloned this region into pKY184 and determined the entire base sequence (Fig. 5). Two ORFs (ORF1 and ORF2) were identified (Fig. 5 and 6) and may constitute an operon, since they are preceded by a promoterlike sequence and followed by a ρ-independent terminator sequence, with ORF1 being proximal.

ORF1 is able to encode a hydrophobic protein of 408 amino acids which may be an integral membrane protein with some 10 transmembrane segments, as predicted by the algorithms of Kyte and Doolittle (23) and Klein et al. (19). ORF2 encodes a protein of 124 amino acid residues, of which 24 are glutamic acid and 14 are aspartic acid; no homologous protein was found by data base searches.

Plasmid pMSY702, which had lost the putative stem-loop terminator structure (Fig. 6), was no longer active, suggesting that the distal part must be intact for suppression to occur. The suppression (at the growth level) required the presence of both ORFs (Fig. 6). However, we reasoned that one of the ORFs could be the suppression determinant and that a plasmid that retained the suppressor but had lost the other ORF might express the suppressor gene poorly because of some altered operon construction. Indeed, pMSY704, which carried only ORF2 (Fig. 6) could significantly improve protein export (Fig. 8). A frameshift mutation in ORF1 (pMSY708) abolished the suppression activity.

Electrophoretic patterns of pulse-labeled (for 2 min) whole-cell proteins (Fig. 7) showed that a protein with a molecular mass of about 25 kDa was overproduced by cells carrying pMSY7, pMSY701, pMSY704, pMSY705, or pMSY708, all of which contained ORF2 (Fig. 6). The overproduction was decreased with pMSY702 with the terminator structure deleted, as well as with pMSY708 containing the ORF1 frameshift mutation that should have caused a polarity effect. OmpA immunoprecipitates from the same samples are shown in the lower part of Fig. 7. Processing kinetics of maltose-binding protein are shown in Fig. 8. These results show a good correlation between the abundance of this protein and the cellular activity of export. We conclude that this protein represents the product of ORF2, which is identical to MsyB. Its apparent molecular weight was higher than the molecular weight expected for the ORF2 product. This may be due to the fact that ORF2 is rich in charged amino acids, especially the acidic ones, a feature often causing insufficient binding of SDS and slow migration in SDS-polyacrylamide gel electrophoresis (10).

DISCUSSION

Suppressor analysis is potentially useful to investigate interactive features of gene products. However, genetic

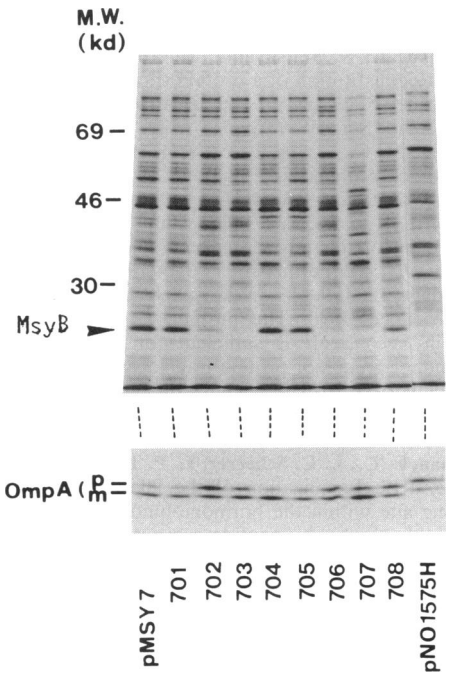

FIG. 7. Pulse-labeled total proteins and OmpA in cells carrying an *msyB* plasmid. IQ85 cells carrying a plasmid as indicated were shifted to 42°C for 2 h and pulse-labeled with [^{35}S]methionine for 2 min. The upper panel shows electrophoretic patterns of total cell proteins, and the lower panel shows OmpA immunoprecipitates. p, precursor; m, mature.

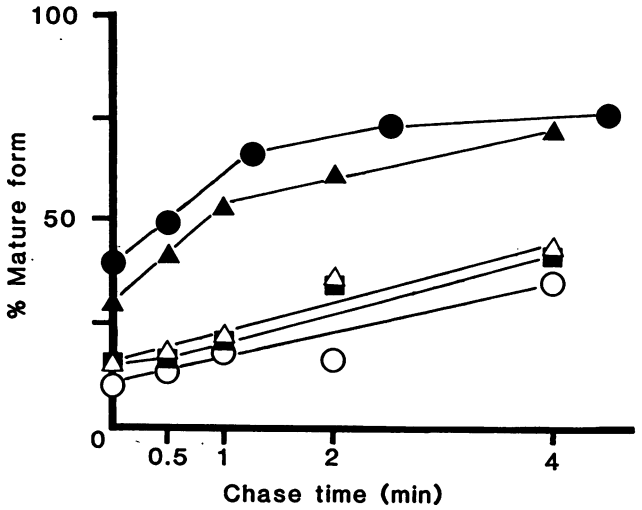

FIG. 8. Kinetics of maltose-binding protein processing in plasmid-bearing *secY24* cells. IQ85 cells carrying a plasmid (○, pNO1575H; ●, pMSY701; △, pMSY702; ▲, pMSY704; ■, pMSY707) were grown with maltose, shifted to 42°C for 2 h, pulse-labeled with [^{35}S]methionine for 0.5 min, and chased with unlabeled methionine for the indicated periods. Maltose-binding protein immunoprecipitates were electrophoresed, autoradiographed, and densitometrically quantitated for proportions of mature form in the total (precursor and mature) species.

suppression can occur by a number of different mechanisms; for example, a suppressor mutation may create a new pathway independent of the primary gene product, may stabilize the mutated primary gene product, or may restore protein-protein interactions that had been impaired by the primary mutation. In the protein export system, the suppressor approach using a signal sequence mutation as the primary mutation led to the discovery of the suppressor alleles of the *prlA* (*secY*), *prlD* (*secA*), and *prlG* (*secE*) genes (7, 41). On the other hand, suppressor isolation against the *secA* or the *secY* temperature-sensitive mutation yielded mutations predominantly in components of the protein-synthesizing machinery (24, 29, 37–39). The basis for the latter results is only poorly understood.

None of the clones isolated in this study as multicopy suppressors of *secY24* overlapped the *ssy* genes (39), which had previously been identified as extragenic suppressors against the same mutation. The fact that these clones did not suppress the *rplO215* mutation suggests that the suppression required the (altered) SecY protein and hence that they did not create a SecY-independent pathway of export. Also, their failure to suppress the *secA51* mutation known to be suppressible by mutations or physiological conditions that lower protein synthesis activity of the cell (24) argues against the possibility that they exert the suppression activities by nonspecifically lowering protein synthesis. Indeed, the *msyA* and *msyB* plasmids did not significantly slow down the growth rate under the conditions where suppression took place. The plasmid that hyperproduced HtpG did exhibit a growth-impairing effect but did not suppress the *secA* mutation. We believe that the multicopy suppression we observed is mechanistically different from the extragenic suppression previously studied. In *Saccharomyces cerevisiae*, a similar approach using a mutation in the *SEC12* gene successfully identified a new gene (*SAR1*) that proved to specify a component involved in the vesicular transport pathway (28).

Entirely different sets of clones were obtained as multicopy suppressors of the *secY24* and the *secA51* mutations. In the latter case, only clones containing the *groE* genes were obtained. In contrast, we did not obtain a *groE* clone as a suppressor of *secY24*. Also, several GroE-overproducing plasmids with different expression levels barely suppressed the *secY* mutation. The reason for the discrepancy between our results and those of Van Dyk et al. (44), who reported that GroE overproducers suppressed both of the *sec* mutations, is unclear.

The multicopy suppressors of *secY24* included HtpG, a heat shock protein homologous to the eukaryotic Hsp90 proteins. No physiological function had previously been assigned for HtpG. Since strains with *htpG* deleted are viable (with a slight growth disadvantage at a high temperature [2]) and capable of active protein export (unpublished results), HtpG cannot be a genuine factor in protein export. The fact that its increased abundance can somehow correct the export defect caused by the *secY* mutation may suggest that HtpG possesses a chaperonelike activity by which it compensates for the mutational defect. Eukaryotic Hsp90 is known to bind to other proteins, such as the steroid hormone receptors (6). Chaperonelike heat shock proteins might have some substrate specificity. GroE, which acts primarily in the cytoplasm, might prefer SecA, which interfaces the cytoplasm and the cytoplasmic membrane, to SecY, which is embedded in the membrane. The present results may suggest that HtpG can act on membrane proteins. Possible interaction between HtpG and SecY should be examined in vitro.

Another class of multicopy suppressors, *msyA*, encodes the H-NS (or H1) histonelike protein. A direct role of this protein in protein export is difficult to envision, since it is tightly associated with the chromosomal DNA in nucleoid

structure. Recently, H-NS's role in global regulation of gene expression became apparent (13). Several mutations that elevate expression of diverse sets of genes proved to be mutations of H-NS (11, 26). Since such mutations are defective alleles of *hns*, it was proposed that H-NS possesses silencer functions for specific sets of genes (11). Alternatively, H-NS, which can specifically bind to the curved regions of DNA (47), may affect gene expression by altering topographical arrangements of the chromatin structure (14). The present study provides a novel phenotype of H-NS that is executed when the protein is overproduced. Although the *Salmonella typhimurium hns* is difficult to clone onto a multicopy plasmid (14), the *E. coli hns* (*msyA*) gene can be cloned onto pBR322-derived vectors (but not onto the pUC plasmids). Suppression of *secY24* occurred at levels of H-NS overproduction that did not appreciably affect cell growth. H-NS overproduction may either silence some gene whose product negatively controls the export activity or activate some gene whose product helps functioning of SecY. It is important to identify proteins that are repressed or induced in response to overproduction of H-NS. We examined whether the *msyA* plasmid affected the expression level of SecY by pulse-labeling and immunoprecipitation experiments but failed to detect any significant effect within the sensitivity limit of the method (data not shown). Thus, *msy*-dependent suppression cannot be attributed to overproduction of the mutated SecY protein.

Finally, the *msy* selection yielded *msyB*, which encodes a highly acidic protein. This gene and a gene presumably specifying a multipath integral membrane protein constitute an operon. The product of the latter gene is hydrophobic as well as basic; such features are expected for transmembrane segments and their cytoplasmic vicinities, respectively (46). Interaction between the acidic *msyB* gene product and the cytoplasmic domains of the ORF1-encoded membrane protein is conceivable. It could be speculated further that MsyB interacts, albeit with low affinity, with SecY, which is similar to the ORF1 product in that it is a multipath membrane protein. Increased abundance of the MsyB protein may make such interaction significant and lead to better functioning of the mutated SecY protein. It is unlikely that the *msyB* gene product directly participates in the normal pathway of protein export, since we could disrupt this region of the chromosome with a drug resistance marker without affecting growth and protein export (unpublished results).

In summary, the multicopy suppressors obtained in this study appear to affect protein export indirectly, pointing to the limitation of this approach. However, it may also be true that studies of *E. coli* are coming to a stage in which gene-phenotype relationships without straightforward interpretation can contribute to the integrated knowledge of this model organism. The *msy* genes could be subjects of future studies concerning the biogenesis of functional export machinery and control of its activity.

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