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Control of Synthesis and Secretion of the *Bacillus subtilis* Protein YqxM

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***yqxM* is a *Bacillus subtilis* gene of unknown function residing in an operon with *sipW*, which encodes a signal peptidase, and *tasA*, which encodes an antibiotic protein secreted in a *sipW*-dependent manner. YqxM was undetectable during growth in a variety of rich media, including Luria-Bertani (LB) medium, or in minimal media or under heat shock or ethanol stress conditions but was synthesized and secreted during growth in LB medium supplemented with 1.2 M NaCl. Consistent with the possible involvement of *sipW* in YqxM secretion, inactivation of *sipW* prevented YqxM secretion. YqxM was produced and secreted in a *sipW*-dependent manner during growth in LB medium when the sequences upstream of *yqxM* were replaced with those of the inducible P_{spac} promoter. Coexpression of *yqxM* and *sipW* in *Escherichia coli* resulted in a decrease in the apparent molecular mass of YqxM, consistent with the removal of a signal peptide. These experiments suggest that YqxM production is induced by a high concentration of salt and that YqxM is secreted under the control of SipW. We hypothesize that during most conditions of growth, YqxM is present at very low levels or is not synthesized at all and that this low level or absence is due, at least in part, to posttranscriptional repression.**

The ability to respond flexibly to a varying environment is essential to bacterial survival. *Bacillus subtilis* can respond to environmental challenges by spore formation, the uptake of foreign DNA (competence), the production of degradative enzymes and antibiotics, or the induction of a large set of general stress proteins (4, 5, 14). In previous work, we identified an operon, expressed during early-stationary-phase growth (12), consisting of *yqxM*, a gene of unknown function, *sipW*, which encodes a signal peptidase (15, 16), and *tasA*, which encodes an antibiotic protein which is secreted at the beginning of sporulation and is also built into the spore (11a, 13) (Fig. 1A). To date, TasA is the only candidate substrate for SipW in *B. subtilis*.

There is no readily observable phenotype when *yqxM* is deleted (13), and the predicted product of *yqxM* does not resemble any other proteins in the databases although it possesses a potential signal peptide at its N terminus (10). To begin to determine the role of YqxM, we have investigated the conditions for YqxM synthesis and secretion.

MATERIALS AND METHODS

General methods and detection of YqxM during growth. Strains, plasmids, and oligonucleotide primers are described in Tables 1 and 2. Media and methods for the growth, sporulation by exhaustion, and genetic manipulation of *B. subtilis* are described in reference 2, and methods for cloning in *Escherichia coli* DH5α are described in reference 11. To identify conditions for the synthesis of YqxM, we grew cells in one of a variety of media for up to 48 h; prepared cell lysates, spore extracts, and culture supernatants; and subjected these to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (13). We then carried out Western blot analysis according to the method described in reference 13, except that 5% dry milk was used as the blocking reagent. Anti-YqxM antibody was used at a dilution of 1:7,500. We used the following media and growth conditions: incubation at 37, 42, or 52°C in Luria-Bertani (LB) medium (11); incubation at 37°C in 2× YT, Terrific broth (11), King's B medium (7) (in which sporulation is inhibited), or Difco sporulation medium (in which the majority of cells sporulate) (data not shown); incubation at 37°C in LB medium supplemented with 0.65, 0.7, 0.8, 1.2, or 1.4 M NaCl or with 2.5 or 10% ethanol; or incubation at 37°C in synthetic minimal medium (1). To analyze the synthesis of YqxM and TasA during growth with high concentrations of salt, we grew cells to stationary phase in LB medium, diluted the culture to an optical density at 600 nm (OD_{600}) of 0.1 in LB medium with 1.2 M NaCl, and continued growth at 37°C. We then prepared cell extracts and concentrated culture supernatants at various times and analyzed them by Western blotting as described above. We used anti-TasA antibodies at a dilution of 1:10,000 (13).

Overproduction of YqxM in *E. coli* and creation of an anti-YqxM antiserum. We used PCR and the primers OL92 and OL91 to generate a DNA fragment beginning 99 nucleotides into the *yqxM* open reading frame (Fig. 1A) (and, therefore, missing most of the sequence encoding the putative signal peptide) and subcloned this fragment into the overexpression plasmid pAGS05 (13), adding six histidine codons to the 3′ end of *yqxM*. We transformed *E. coli* BL21(DE3) with the resulting plasmid (pAGS36), induced expression with IPTG (isopropyl-β-D-thiogalactopyranoside) according to the directions supplied by Novagen, and prepared overproduced protein as described previously (13). We then lysed the cells by passing them twice through a French press (at 18,000 lb/in^2), isolated the overproduced YqxM from the lysate by nickel chromatography using His-Bind resin (Novagen), and injected about 200 μg of the purified material into rabbits (3).

P_{spac}-driven expression of the locus. To place *yqxM*, *sipW*, and *tasA* under the control of the inducible P_{spac} promoter (18), we first used PCR and the primers OL116 and OL115 to create a fragment of DNA beginning at the first codon of the *yqxM* open reading frame and ending 67 bp 3′ of *yqxM* (Fig. 1A). We digested the PCR product and pAG58 (6) with *Nhe*I and *Sph*I and ligated these DNA fragments. We introduced the resulting plasmid (pAGS52) into the *B. subtilis* genome by Campbell-type single reciprocal integration (2) at *yqxM*, creating strain AGS339. This operation placed all three genes of the operon under the direction of P_{spac} and separated the locus from potential upstream regulatory sequences (Fig. 1B). To delete *sipW* from strain AGS339 by marker replacement, we transformed this strain with linearized pAGS17-2 (13) (Fig. 1B). We confirmed both integration events using PCR analysis (data not shown). We induced P_{spac}-driven expression of *yqxM*, *sipW*, and *tasA* by the addition of 1 mM IPTG to cells during exponential-phase growth (after culturing in LB medium for 3 h), and prepared cell extracts as described previously (13).

Overproduction of YqxM in *E. coli* to test secretion and processing. To overproduce YqxM or YqxM and SipW in *E. coli* (for the experiment whose results are shown in Fig. 4), we used PCR and the primers OL99 and OL91 or primers OL99 and OL110 to generate DNA fragments beginning at the first nucleotide of the *yqxM* open reading frame and ending at the last nucleotide of *yqxM* or ending 68 nucleotides downstream of *sipW*, respectively (Fig. 1A). We digested these fragments with *Nde*I and *Xho*I or with *Nde*I and *Not*I, respectively, subcloned them into appropriately digested pAGS05 or pET24b, respectively, and used the resulting plasmids to transform BL21(DE3). We induced expression of *yqxM* or *yqxM* and *sipW* by the addition of 1 mM IPTG for 0.75 h of growth in LB medium at 37°C. We then prepared cell extracts and concentrated culture supernatants, fractionated them by SDS-PAGE using 12% polyacrylamide gels, and performed Western blot analysis (13). In lanes 1 to 12 of Fig. 4, we loaded

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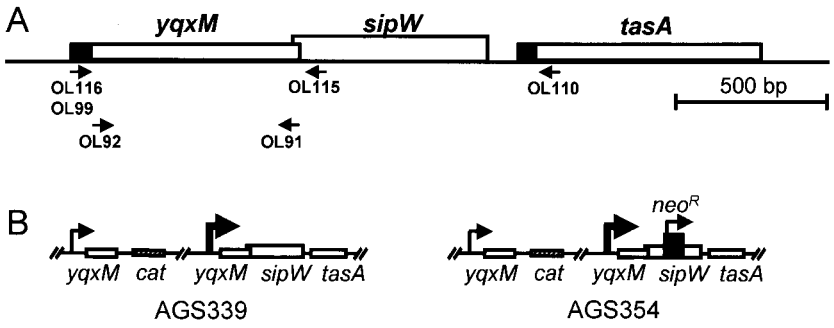

FIG. 1. *yqxM sipW tasA* locus and P_{spac}-driven constructs. (A) The open boxes indicate genes, and the closed boxes at the 5′ ends of *yqxM* and *tasA* indicate potential signal peptide sequences. The directions of transcription are from left to right. Arrows underneath the genes indicate the binding sites of primers used in this study. (B) On the left, *yqxM*, *sipW*, and *tasA* are under the control of P_{spac} (thick arrow), as in strain AGS339. On the right, *sipW* has been inactivated by the introduction of a neomycin resistance gene (*neo), resulting in strain AGS354. The thin arrows indicate the likely locations of an endogenous promoter (in front of yqxM*), and the arrow above the neomycin resistance gene indicates its promoter and direction of transcription. The plasmid-borne *cat* marker is shown.

an amount of cell extract corresponding to 0.5 OD_{600} unit of the original culture, and in lanes 13 to 24, we loaded 200 μl of ethanol-precipitated culture supernatant. We used anti-acetyl coenzyme A (acetyl-CoA) synthetase antibody, the kind gift of Alan Wolfe, at a dilution of 1:2,000. We used nucleotide sequencing to confirm that pAGS65 had no frameshift or nonsense mutations (data not shown).

RESULTS

YqxM synthesis and secretion during growth with high concentrations of salt. Surprisingly, we did not detect YqxM in cell lysates, spore extracts, or culture supernatants of *B. subtilis* grown under a wide variety of conditions, including growth in rich and minimal media and under heat and ethanol stress (see Materials and Methods and data not shown). However, we did detect YqxM in the culture supernatant, but not in cell extracts, during growth in LB medium supplemented with 0.65 to 1.2 M NaCl (results of growth with 1.2 M NaCl are shown in Fig. 2). YqxM first became detectable by Western blotting about 1 h prior to stationary phase and was present for at least 4 h (Fig. 2A and data not shown). We found that TasA became detectable at about the same time, as seen previously during growth in media with the standard concentration of NaCl (13) (data not shown). Under these conditions, YqxM migrated as a protein of approximately 38 kDa, similar to its apparent molecular mass when it was overproduced in *E. coli* (data not shown) but larger than the mass of about 24.5 kDa predicted from the sequence. Growth of the *yqxM* mutant strain (AGS175 [13]) was similar to that of the wild type during culture in either LB medium or LB medium with 1.2 M NaCl (13) (data not shown).

To learn whether *sipW* was required for YqxM secretion, we used Western blot analysis and anti-YqxM antibodies to examine cell extracts and culture supernatants prepared 16 h (Fig. 2B) after the beginning of stationary-phase growth, with LB medium containing 1.2 M NaCl. We found that the secretion of YqxM depended on *sipW* (Fig. 2B, compare lanes 2 and 6). We did not detect YqxM in extracts of *sipW* cells, suggesting that nonsecreted YqxM is rapidly proteolyzed.

P_{spac}-driven *yqxM* expression permits YqxM synthesis. The finding that YqxM synthesis is inhibited during growth conditions that permit expression of the operon (such as growth in LB medium [12]) suggested either that an upstream translational inhibitory sequence is present or that the level of *yqxM* message was insufficient for significant protein synthesis. To bypass both potential mechanisms, we placed *yqxM*, *sipW*, and *tasA* under the control of the inducible promoter P_{spac} (18), in a manner that uncoupled *yqxM* from the endogenous upstream sequences (in strains AGS339 and AGS354) (Fig. 1B). We then used Western blot analysis to determine the steady-state levels of YqxM and, as a control, TasA in these cells, when the cells were grown in LB medium. As expected from previous results, we found TasA in the cell extract and culture supernatants when IPTG was present (Fig. 3, lanes 2 and 10) (13). The secreted form of TasA migrated more slowly than TasA in cell extracts, possibly an effect of the culture supernatant on electrophoresis. We also detected YqxM in culture supernatants of these cells and, in contrast to what occurred with

TABLE 1. Strains and plasmids

Strain or Plasmid (species)	Description[a]	Reference, source, or construction
Strains		
PY79 (*B. subtilis*)	Wild type	19
AG518 (*B. subtilis*)	*abr*::Tn*917 trpC2 pheA1*	A. D. Grossman
AG839 (*B. subtilis*)	Δ*abr::cat trpC2 pheA1*	A. D. Grossman
AGS157 (*B. subtilis*)	*sipW*Δ::$\overleftarrow{neo}$	13
AGS175 (*B. subtilis*)	yqxM::$\overrightarrow{neo}$	13
AGS339 (*B. subtilis*)	*yqxM*ΩpAGS52	This study
AGS347 (*B. subtilis*)	*abrB*::Tn*917*	PY79 × DNA AG518
AGS348 (*B. subtilis*)	Δ*abrB*::*cat*	PY79 × DNA AG839
AGS354 (*B. subtilis*)	*yqxM*ΩpAGS52 Δ*sipW*::$\overrightarrow{neo}$	AGS339 × pAGS17-2
AGS40 (*E. coli*)	BL21(DE3)/pET24b	This study
AGS249 (*E. coli*)	BL21(DE3)/pAGS36	This study
AGS253 (*E. coli*)	BL21(DE3)/pAGS05	This study
AGS275 (*E. coli*)	BL21(DE3)/pAGS41	This study
AGS406 (*E. coli*)	BL21(DE3)/pAGS65	This study
DH5α (*E. coli*)	Cloning host	Lab collection
BL21(DE3) (*E. coli*)	Overproduction host	Lab collection
Plasmids		
pAG58	P_{spac} expression plasmid	6
pAGS05	Overexpression plasmid	13
pAGS17-2	Insertion deletion in *sipW*	13
pAGS36	T7 control of proless *yqxM*	This study
pAGS41	T7 control of *yqxM*	This study
pAGS52	P_{spac} control of *yqxM*	This study
pAGS65	T7 control of *yqxM* and *sipW*	This study
pET24b	Overexpression plasmid	Novagen

[a] Arrows above gene names indicate directions of transcription.

TABLE 2. Primers[a]

Oligonucleotide	Sequence	Restriction endonuclease(s)	Nucleotide positions
OL91	5′ AAAAAAAAACTCGAGCTGATCAGCTTCATTGCT 3′	*Xho*I	741–758
OL92	5′ AAAAAAAAACATATGTGCTTACAATTTTTC 3′	*Nde*I	99–114
OL99	5′ AAAAACTCGAG**CATATG**TTTCGATTGTTTCAC 3′	*Xho*I/*Nde*I	1–18
OL110	5′ AAAAAAGCGGCCGCCCTCCAACTAAAGCTAATCCTAGTG 3′	*Not*I	1424–1448
OL115	5′ AAAAAAGCGGCC**GCATGC**TGAACGTGTTGAAATCACGAC 3′	*Not*I/*Sph*I	808–818
OL116	5′ AAAAAAGCTAGCATGTTTCGATTGTTTCAC 3′	*Nhe*I	1–18

[a] The restriction endonuclease site(s) in each oligonucleotide is underlined (or in boldface), and the enzyme that cuts it is listed. For OL99, the *Xho*I site is underlined and the *Nde*I site is in boldface, and for OL115, the *Not*I site is underlined and the partially overlapping *Sph*I site is in boldface. The region of the chromosome bound by each oligonucleotide is indicated as nucleotide positions relative to the start site of the *yqxM* open reading frame (9).

wild-type cells grown with high salt, in cell extracts (Fig. 2B, lane 1; Fig. 3, lanes 14 and 6). YqxM migrated as a protein of approximately 30 kDa, 8 kDa smaller than in cells grown with high salt or during overproduction in *E. coli*, possibly due to the action of a protease. The electrophoretic mobilities of YqxM in culture supernatants and in cell extracts were similar, suggesting that if YqxM is not secreted, it is cleaved at a position similar to that of secreted YqxM, towards the N terminus.

It was possible that P_{spac}-driven expression of *yqxM* produced a protein due to an elevated level of message. To test this possibility, we analyzed strains bearing mutations in *abrB* (AGS347 and AGS348) which result in an approximately 15-fold derepression of *tasA* transcription (12). We did not detect YqxM in extracts of these cells by Western blot analysis (data not shown), which argues that increased transcription alone is probably insufficient to permit the appearance of YqxM. More likely, under most growth conditions, a posttranscriptional event represses the synthesis of YqxM. Although we have no data regarding ribosome binding, the Shine-Dalgarno sequence associated with *yqxM* is in perfect agreement with the *B. subtilis* consensus sequence (17).

To determine whether YqxM secretion is *sipW* dependent under these conditions of synthesis, we expressed *yqxM* in the absence of *sipW* (in strain AGS354) (Fig. 1B) and then used Western blot analysis to determine whether YqxM and, as a control, TasA, were present in extracts and supernatants of these cells. In this construct, *tasA* expression is constitutive (13) (Fig. 3, lanes 3 and 4). Consistent with the loss of SipW function, we detected the immature form of TasA in extracts and very little TasA in the supernatant (Fig. 3, lanes 3, 4, 11, and 12) (13). We observed YqxM in extracts of strain AGS354 but not in the culture supernatant (Fig. 3, lanes 7, 8, 15, and 16). Unexpectedly, YqxM was present in the *sipW* mutant strain when cells were grown without IPTG as well as with IPTG (Fig. 3, lanes 7 and 8). The reason for this result is unknown.

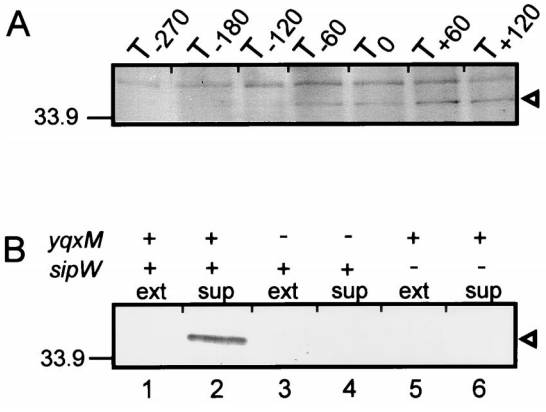

FIG. 2. Synthesis of YqxM during growth with high salt. (A) Wild-type cells (PY79) were grown in LB medium with 1.2 M NaCl. Culture at an OD_{600} of 0.2 was harvested at the indicated times ([T] in minutes) before or after the onset of stationary-phase growth, fractionated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and probed with anti-YqxM antibodies. The position of YqxM is indicated by an arrowhead. (B) Wild-type cells (PY79, lanes 1 and 2), *yqxM* mutant cells (AGS175, lanes 3 and 4), or *sipW* mutant cells (AGS157, lanes 5 and 6) were grown in LB medium with 1.2 M NaCl, and cell extracts (ext) (lanes 1, 3, and 5) and culture supernatants (sup) (lanes 2, 4, and 6) were prepared 16 h after the onset of stationary-phase growth. After fractionation by SDS-PAGE and transfer to PVDF membranes, blots were probed with anti-YqxM antibodies. The position of YqxM is indicated by an arrowhead. Molecular mass is indicated at the left in kilodaltons.

The above-described experiments established the dependency of YqxM secretion on *sipW* but did not permit us to visualize the expected change in YqxM mobility that should result from the cleavage of a signal peptide. Therefore, we examined SipW-dependent YqxM cleavage in *E. coli*. In cell extracts from an *E. coli* strain producing YqxM alone (AGS275), we detected a band of about 38 kDa as well as an approximately 33-kDa band that we suspect is the result of proteolytic cleavage (Fig. 4, lane 5). The presence of these bands in the absence of IPTG suggested some promoter activity even without IPTG (Fig. 4, lanes 2 and 3). In extracts from cells expressing *yqxM* and *sipW* (AGS406), we observed a band of approximately 35 kDa (Fig. 4, lane 3 and 6). This indicated that *sipW* was responsible for a decrease in the molecular mass of YqxM, consistent with the cleavage of the putative signal peptide. Presumably, the 35-kDa species had been translocated into the periplasm. The slight difference in mobility between the apparently secreted forms of YqxM in *E. coli* and in *B. subtilis* may reflect effects of the different cell extracts and supernatant on electrophoresis or different proteases in the two organisms.

We found some YqxM in the *E. coli* culture supernatant (Fig. 4, lanes 17 and 18). We hypothesized that this was the result of cell lysis and not translocation across the outer membrane. Consistent with this view, the levels of the cytoplasmic enzyme acetyl-CoA synthetase (8) (Fig. 4, lanes 23 and 24) reflected the levels of YqxM in the culture supernatant (Fig. 4, lanes 17 and 18).

DISCUSSION

Our results indicate that during growth with high salt, YqxM is produced and, in a *sipW*-dependent manner, secreted. Previously, we showed that the *yqxM sipW tasA* operon is transcribed during postexponential-phase growth (12), resulting in the SipW-dependent secretion of the antibacterial protein TasA (13). It seems likely that TasA provides a competitive advantage to cells in the soil during conditions of nutrient limitation. The present study suggests an additional role for the

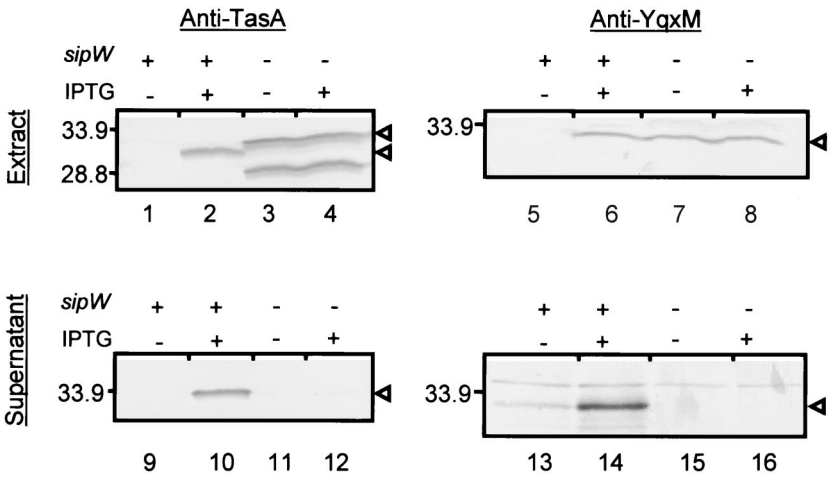

FIG. 3. Western blot analysis of YqxM and TasA in cells with P_{spac}-induced synthesis. Cells with *yqxM*, *sipW*, and *tasA* expression under the control of P_{spac} (AGS339, lanes 1, 2, 5, 6, 9, 10, 13, and 14) or with *yqxM* under the control of P_{spac}, a mutation in *sipW*, and constitutive *tasA* expression (AGS354, lanes 3, 4, 7, 8, 11, 12, 15, and 16) were grown with IPTG (lanes 2, 4, 6, 8, 10, 12, 14, and 16) or without IPTG (lanes 1, 3, 5, 7, 9, 11, 13, and 15), and cell extracts (lanes 1 to 8) and culture supernatants (lanes 9 to 16) were prepared. After fractionation by SDS-PAGE and transfer to PVDF membranes, blots were probed with anti-YqxM (lanes 5 to 8 and 13 to 16) or anti-TasA (lanes 1 to 4 and 9 to 12) antibodies. The arrowheads adjacent to lane 4 indicate the immature (upper arrowhead) and the mature (lower arrowhead) forms of TasA. The arrowhead adjacent to lane 12 indicates the form of TasA found in the supernatant. The arrowheads adjacent to lanes 8 and 16 indicate YqxM. Molecular masses are indicated in kilodaltons.

operon in adaptation to high levels of salt. The function of YqxM is unknown, but the lack of an obvious growth defect in *yqxM* mutant cells during growth with high salt argues against a direct role in protection from salt stress. Possibly, YqxM enables the cells to survive some additional environmental challenge concomitant with this stress in nature, much as we argue for TasA (13). An intriguing possibility is that YqxM is an antibiotic designed to function in the presence of elevated concentrations of salt.

We do not know how the 5′ end of the *yqxM sipW tasA* transcript participates in inhibition of YqxM synthesis during growth in rich media. Possibly, this portion of the message forms a structure or binds a protein that blocks access of the ribosome to the *yqxM* translational initiation site. Inhibition may be overridden by a posttranscriptional event that frees the translational initiation site or by use of an alternative transcriptional start site.

Our work also indicates that SipW can function in *E. coli*, an organism only distantly related to *B. subtilis*. This finding reinforces the notion that no additional specialized factors are

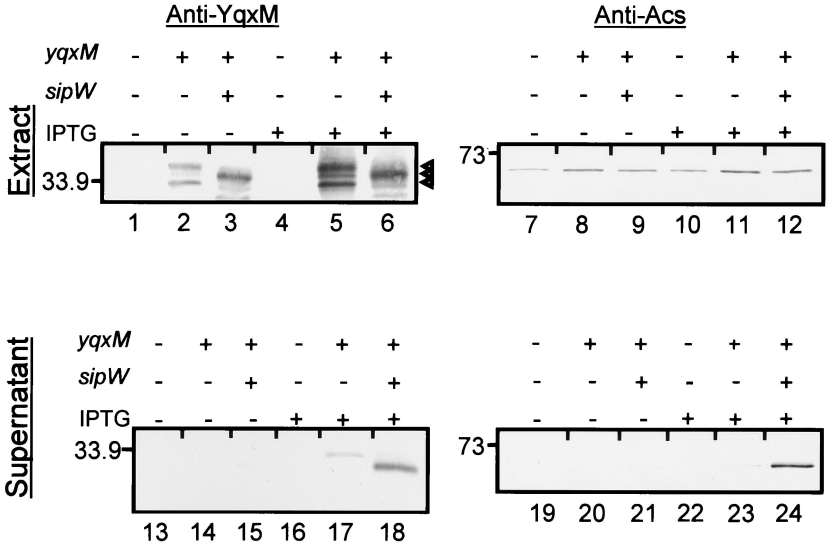

FIG. 4. Western blot analysis of YqxM in *E. coli*. *E. coli* engineered to overproduce either YqxM (AGS275, lanes 2, 5, 8, 11, 14, 17, 20, and 23), YqxM and SipW (AGS406, lanes 3, 6, 9, 12, 15, 18, 21, and 24), or no protein (AGS40, lanes 1, 4, 7, 10, 13, 16, 19, and 22) was grown without IPTG (lanes 1 to 3, 7 to 9, 13 to 15, and 19 to 21) or with IPTG (lanes 4 to 6, 10 to 12, 16 to 18, and 22 to 24), and cell extracts (lanes 1 to 12) and culture supernatants (lanes 13 to 24) were prepared. In each of lanes 1 to 8, we loaded an amount of cell extract corresponding to 0.5 OD_{600} unit of the original culture. For lanes 9 to 16, we loaded 200 μl of ethanol-precipitated culture supernatant (13). These preparations were subjected to electrophoresis on 12% polyacrylamide gels and Western blot analysis as described above. Samples were fractionated by SDS-PAGE, transferred to PVDF membranes, and probed with anti-YqxM or anti-acetyl-CoA synthetase (Anti-Acs) antibodies. The three arrowheads adjacent to lane 6 indicate, from top to bottom, the immature form, the mature form, and a proteolytic product of YqxM. Molecular masses are indicated at the left of the gels in kilodaltons.

required for SipW-dependent cleavage and further suggests that SipW participates in a mechanism of export that is relatively well conserved across species. The ability to study SipW activity and substrate specificity in a heterologous system may be advantageous in dissecting this secretory pathway and raises the intriguing question of whether SipW-like enzymes exist in *E. coli*. This study also demonstrates an approach for studying poorly characterized potential substrates of signal peptidases. By placing the gene for such a protein under the control of P_{spac}, the mechanism of secretion can be studied without any knowledge of the normal circumstances for its expression.

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