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Functional Identification of the Product of the *Bacillus subtilis yvaL* Gene as a SecG Homologue

KAREL H. M. VAN WELY, JELTO SWAVING, CEES P. BROEKHUizen, MATTHIAS ROSE, WIM J. QUAX, and ARNOLD J. M. DRIESSEN

Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9751 NN Haren, Genencor International, 2600 AP Delft, and Department of Pharmaceutical Biology, University of Groningen, 9713 GZ Groningen, The Netherlands, and Institut für Mikrobiologie, Johann-Wolfgang-Goethe-Universität Frankfurt, D 60439 Frankfurt am Main, Germany

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*Bacillus subtilis*, a gram-positive bacterium, has arisen next to *Escherichia coli* as a paradigm for studies on protein secretion primarily because bacilli have a high capacity for the production of exoenzymes. Protein secretion across the cytoplasmic membrane of *B. subtilis* is thought to be catalyzed by a system that is homologous to the precursor protein translocase of *E. coli* (22, 34). In *E. coli*, precursor protein translocation is mediated by a cytosolic chaperone, SecB; the translocation ATPase, SecA; and a large integral membrane protein complex with SecY, SecE, and SecG (9). SecD and SecF are accessory subunits that are not essential for translocation but that associate with SecY, SecE, and SecG (9). SecD and SecF are ATPase, SecA; and a large integral membrane protein complex with SecY, SecE, and SecG, respectively. The analogous genes of a *secG* homologue in *B. subtilis* have been found in other gram-negative bacteria, but none have been demonstrated in gram-positive bacteria. Since the *secG* codes for a nonessential component of the precursor protein translocase, its genetic identification is complicated. Recently, the sequencing of the *B. subtilis* chromosome has been completed (17). We now report on the identification of an open reading frame, *yvaL*, that bears significant sequence similarity to the *E. coli secG* gene. Our data demonstrate that *yvaL* codes for a protein that is functionally homologous to SecG.

Protein export in *Escherichia coli* is mediated by translocase, a multisubunit membrane protein complex with SecA as the peripheral subunit and the SecY, SecE, and SecG proteins as the integral membrane domain. In the gram-positive bacterium *Bacillus subtilis*, SecA, SecY, and SecE have been identified through genetic analysis. Sequence comparison of the *Bacillus* chromosome identified a potential homologue of *SecG*, termed *YvaL*. A chromosomal disruption of the *yvaL* gene results in mild cold sensitivity and causes a β-lactamase secretion defect. The cold sensitivity is exacerbated by overexpression of the secretory protein α-amylase, whereas growth and β-lactamase secretion are restored by coexpression of *yvaL* or the *E. coli secG* gene. These results indicate that the *yvaL* gene codes for a protein that is functionally homologous to SecG.
TABLE 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Replicon</th>
<th>Resistance(s)</th>
<th>Relevant expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDELG2</td>
<td>ColE1</td>
<td>Amp’ Cam’</td>
<td>—</td>
</tr>
<tr>
<td>pPR111</td>
<td>ColE1, repR</td>
<td>Amp’ Phe’</td>
<td>—</td>
</tr>
<tr>
<td>pET470</td>
<td>ColE1, repR</td>
<td>Amp’ Phe’</td>
<td>E. coli SecG</td>
</tr>
<tr>
<td>pET471</td>
<td>ColE1, repR</td>
<td>Amp’ Phe’</td>
<td>B. subtilis YvaL</td>
</tr>
<tr>
<td>pET468</td>
<td>repA</td>
<td>Ery’</td>
<td>AmyQ</td>
</tr>
<tr>
<td>pET472</td>
<td>repA</td>
<td>Ery’</td>
<td>E. coli SecG</td>
</tr>
<tr>
<td>pET473</td>
<td>repA</td>
<td>Ery’</td>
<td>AmyQ, B. subtilis YvaL</td>
</tr>
<tr>
<td>pET304</td>
<td>ColE1</td>
<td>Amp’</td>
<td>E. coli SecG</td>
</tr>
<tr>
<td>pET820</td>
<td>ColE1</td>
<td>Amp’</td>
<td>B. subtilis YvaL</td>
</tr>
</tbody>
</table>

“—”, deletion vector.

MATERIALS AND METHODS

Bacterial strains and growth media. Strains were grown in Luria-Bertani broth or on Luria-Bertani agar. When necessary, the medium was supplemented with relevant antibiotics as indicated. Construction of vectors was done with E. coli DH5α [supE44 ΔlacU169 (Δ80lacZΔM15) thi-1 endA1 gyr96 thi-1 relA1] and B. subtilis DB104, respectively, and cloned into pBluescript SK+ by using the primers listed in Table 2. The sequences of both open reading frames were determined and compared against the relevant databases. For expression in E. coli, the genes were cloned into pET324 (31), yielding pET304 (E. coli secG) and pET820 (B. subtilis yvaL).

Construction of plasmids. All of the relevant plasmids are listed in Table 1. The E. coli secG and B. subtilis yvaL genes, including suitable ribosome binding sites, were amplified as BamHI-XbaI cassettes by PCR from chromosomal DNA of strains DH5α and DB104, respectively, and cloned into pET8123, using the primers listed in Table 2. The sequences of both open reading frames were determined and compared against the relevant databases. For expression in E. coli, the genes were cloned into pET324 (31), yielding pET304 (E. coli secG) and pET820 (B. subtilis yvaL).

Vectors pPR111, a pUB110 derivative (7), and pBEY3 a gift from R. Breitling (4a), are shuttle vectors using a ColE1 origin for replication in E. coli and repB for replication in gram-positive organisms. These plasmids encode ampicillin resistance (Amp’) markers for E. coli and phleomycin resistance (Phe’) markers for B. subtilis. Vector pBEY3 expresses the B. subtilis secE and secG genes from the constitutive staphylococcal sac promoter. Plasmids pET470 and pET471 were formed by replacing the secE2 cassette with E. coli secG and B. subtilis yvaL, respectively.

Vector pPAMP21 is a pGK13 (14)-based broad-host-range vector containing a ColE1 origin of replication for replication in E. coli and repB for replication in gram-positive organisms. These plasmids encode ampicillin resistance (Amp’) markers for E. coli and phleomycin resistance (Phe’) markers for B. subtilis. Vector pAMP21 was constructed from pPAMP21 by replacing the ampicillin resistance (Amp’) marker with the phleomycin resistance (Phe’) marker.

Disruption of the yvaL gene. The yvaL gene was disrupted in B. subtilis DB104 as follows. Regions immediately upstream and downstream of yvaL were amplified from chromosomal DNA from strain DB104 as BamHI-XbaI and XhoI-HindII cassettes, respectively, and cloned into pBluescript SK+. Subsequently, a BglII-PvuII digested chloramphenicol resistance (Cam’) marker was placed between the BamHI and HindII sites, yielding pDELG2. This vector contains the DB104 chromosomal region with the yvaL gene replaced with the Cam’ marker. Vector pDELG2 was linearized with PvuII to yield a 2.8-kb fragment containing the yvaL::cam region and subsequently transformed into B. subtilis DB104 by natural competence (36). Cam’ colonies resulting from a double crossover were selected. The correct position of the chromosomal replacement was confirmed by PCR. In the resulting strain, DB104::yvaL, the Cam’-encoding gene replaced the yvaL gene while leaving the flanking regions intact. Since the mutations cause a complete deletion, no selective pressure is needed after the initial selection.

TABLE 2. PCR amplification primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (restriction enzyme)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. amylophilaeaeifexis amiO</td>
<td>GGCCATGTTCAAAAACGAAAAACG (NcoI)</td>
</tr>
<tr>
<td>B. amylophilaeaeifexis amiO</td>
<td>GGCCATGTTCAAAAACGAAAAACG (NcoI)</td>
</tr>
<tr>
<td>B. subtilis yvaL forward</td>
<td>AAAGATCTCTGTCGGTGGATGTTAGGGATGC (BamHI)</td>
</tr>
<tr>
<td>B. subtilis yvaL reverse</td>
<td>AAATACGAGCTTGCACCGCTCATAGGAATGGAC (XbaI)</td>
</tr>
<tr>
<td>E. coli secG forward</td>
<td>AACCTCTAGATTGACCATTAATTCGATGTAACG (BamHI)</td>
</tr>
<tr>
<td>E. coli secG reverse</td>
<td>CCCGTAGATCGGTATTGCGTATACG (XbaI)</td>
</tr>
</tbody>
</table>

* Recognition sites of the restriction enzymes used are underlined. Ribosome binding sites and start and stop codons are in boldface.

B. subtilis DB104 and DB104::yvaL were grown overnight at 30°C in liquid medium. The overnight cultures were diluted 1:50 into fresh medium and grown to an optical density at 600 nm (OD600) of 0.6, at which point 1 mM IPTG was added and growth was allowed to resume for another 3 h. Cells were harvested by centrifugation, resuspended in TN buffer (25 mM Tris-Cl [pH 7.5], 10 mM NaCl), and subjected to French pressure treatment (three times at 5,000 l/mm²). Cells debris was removed by centrifugation at 10,000 × g for 10 min, and vesicles were collected by centrifugation at 150,000 × g for 45 min. Vesicles were suspended in TN buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using antibodies directed against SecG and YvaL.

Analysis of cellular and secreted proteins. B. subtilis DB104 and DB104::yvaL were grown overnight at 30°C in liquid medium. The overnight cultures were diluted 1:50 into fresh medium and grown to mid-logarithmic phase at different temperatures. Cultures were cooled on ice and fractionated into cellular and medium fractions by centrifugation. The medium fraction was precipitated with 10% (wt/vol) (final concentration) trichloroacetic acid, washed twice with cold acetone, and analyzed by SDS-PAGE. Cellular pellets were resuspended in sample buffer, sonicated, and analyzed by SDS-PAGE.

RESULTS

Identification of a secG homologue in B. subtilis. The Subtilist database of the B. subtilis chromosome was scanned with the E. coli secG gene by using the Blast search program included in reference 10a. This search yielded the yvaL gene (accession no. BG14067) as the only likely candidate (Fig. 1), with an E value of 2.4 × 10⁻⁵. The E value of the next best score was 0.65. YvaL is a 228-bp gene located at 295° of the genetic map of the B. subtilis chromosome (Fig. 2), in a region that bears many

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</thead>
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</tr>
<tr>
<td>B. subtilis yvaL forward</td>
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</tr>
<tr>
<td>B. subtilis yvaL reverse</td>
<td>AAATACGAGCTTGCACCGCTCATAGGAATGGAC (XbaI)</td>
</tr>
<tr>
<td>E. coli secG forward</td>
<td>AACCTCTAGATTGACCATTAATTCGATGTAACG (BamHI)</td>
</tr>
<tr>
<td>E. coli secG reverse</td>
<td>CCCGTAGATCGGTATTGCGTATACG (XbaI)</td>
</tr>
</tbody>
</table>
genes whose functions are unknown. yvaL seems to be the first gene in an operon, since the upstream gene yvaM is transcribed from the opposite strand. Downstream of yvaL, five genes can be identified without obvious promoter or terminator sequences in between but followed by a clear terminator structure.

YvaL codes for an integral membrane protein of 76 amino acids that, in analogy to SecG, is predicted to span the membrane twice. It is 33% identical and 57% similar to E. coli SecG. Further searches with the B. subtilis yvaL and E. coli secG genes using the Blast server at reference 18a revealed the presence of homologues in other gram-positive bacteria. A multiple sequence alignment of these putative SecG proteins is shown in Fig. 1. Overall, the putative SecG homologues of gram-positive bacteria appear to be shorter than their counterparts in gram-negative bacteria. Although the Mycobacterium leprae secG gene is indicated in the data banks as such, no functional evidence is available that this open reading frame is, indeed, functionally homologous to SecG.

Deletion of yvaL causes mild cold sensitivity of growth. Disruption of the secG gene has been shown to result in a cold-sensitive phenotype of E. coli MC4100-derived strains (20) at temperatures of 25°C and below. Assuming that SecG and YvaL function in the same manner, deletion of yvaL is expected to render B. subtilis cold sensitive as well. Therefore, the yvaL gene was deleted completely from the chromosome of B. subtilis DB104 by homologous recombination and replaced with a Cam' marker. The correct position of the chromosomal replacement was confirmed by PCR. The resulting strain, DB104ΔyvaL, was normally viable at 37°C when grown on either rich or minimal medium. Incubation below 20°C revealed mild cold sensitivity, and the strain showed progressively slower growth than DB104 (data not shown). The cold-
sensitive phenotype is not absolute. Compared to that of the wild type, growth was retarded more severely when the temperature was further lowered, but after the cells were shifted again to higher temperatures, growth resumed at a rate comparable to that of the wild type.

To analyze in more detail the phenotype of the deletion strain compared to that of the wild type, cells were transformed with plasmids expressing *E. coli* SecG or *B. subtilis* YvaL, as well as a control plasmid (Table 3). After preincubation at temperatures that do not affect the growth of the deletion strain, cells were plated and incubated at various temperatures. Growth of the colonies was monitored over a period of several days. Wild-type cells were not affected, and mutant cells transformed with the control plasmid behaved like their non-transformed counterparts, showing retarded growth but not a complete stop at lower temperatures. Transformation of the deletion strain with pET471 expressing the *yvaL* gene product relieved the retardation of growth, showing that the phenotype of the mutant was caused not by any polar effects but by the deletion of *yvaL* alone. Surprisingly, when the mutant was transformed with pET470 expressing *E. coli* SecG, growth stopped completely at temperatures of 20°C or lower. Also in wild-type cells, expression of *E. coli* SecG caused some interference with growth at low temperatures, possibly due to competition for SecYE with YvaL. These data indicate that disruption of *yvaL* from the *B. subtilis* chromosome causes mild cold sensitivity of growth. However, the effect is much weaker than that reported for *E. coli* KN370 (20).

Cold sensitivity of the growth of a *B. subtilis ΔyvaL* strain is exacerbated by overexpression of preAmyQ. Since no complete cold sensitivity could be demonstrated for the DB104ΔyvaL strain, cells were transformed with high-copy plasmid PET468 and derivatives. These plasmids express the precursor form of α-amylase (preAmyQ) to high levels, thereby invoking secretory stress. Derivatives pET472 and pET473 express preAmyQ in combination with SecG or YvaL, respectively. Expression of preAmyQ did not retard the growth of the deletion mutant at 30°C, the temperature used to preculture the cells. The level of secreted α-amylase was the same for the wild type and the deletion mutant, as judged by halo formation on starch-containing plates and analysis of culture supernatants (data not shown). When pET468 transformants of *B. subtilis* DB104ΔyvaL were shifted to lower temperatures, clear and complete cold sensitivity was evident. Already at 20°C, cells stopped growing.

---

**TABLE 3. Results of growth experiments**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Expression</th>
<th>Growth* at:</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15°C</td>
<td>20°C</td>
<td>25°C</td>
<td></td>
</tr>
<tr>
<td>DB104::111</td>
<td></td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DB104::470</td>
<td><em>E. coli</em> SecG</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>DB104::471</td>
<td><em>B. subtilis</em> YvaL</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ΔyvaL::111</td>
<td></td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>ΔyvaL::470</td>
<td><em>E. coli</em> SecG</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>ΔyvaL::471</td>
<td><em>B. subtilis</em> YvaL</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>DB104::468</td>
<td>α-Amylase</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>DB104::472</td>
<td>α-Amylase, <em>E. coli</em> SecG</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>DB104::473</td>
<td>α-Amylase, <em>B. subtilis</em> YvaL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ΔyvaL::468</td>
<td>α-Amylase</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>ΔyvaL::472</td>
<td>α-Amylase, <em>E. coli</em> SecG</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>ΔyvaL::473</td>
<td>α-Amylase, <em>B. subtilis</em> YvaL</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

* Growth was scored as follows: ++, growth like that of the reference; ±, growth slower than that of the reference; --, no growth.

---

FIG. 3. Coomassie-stained SDS-PAGE of cellular and medium fractions of *B. subtilis* DB104 and DB104ΔyvaL cultures grown to mid-logarithmic phase at 37°C (A) or 20°C (B). Positions of 30- and 120-kDa proteins absent in the supernatant of the *yvaL* mutant are indicated.
completely (Table 3), and when the bacteria were transferred back to the permissive temperature of 30°C after prolonged incubation at 20°C, growth was not resumed. Apparently, the deletion mutant is capable of sustaining a basic level of secretion even at lower temperatures but cannot handle the overexpression of a secretory protein within a broad temperature range. When coexpressed, both secG and yvaL complemented the deletion mutant, albeit the growth level of the transformants did not reach that of the wild type. Also, in this case, expression of SecG, but not that of YvaL, interferes with the growth of the wild type in a temperature-dependent way.

**Effect of the yvaL deletion on the secretion of proteins.** To investigate more directly the involvement of the precursor protein translocase in the cold-sensitive phenotype of the ΔyvaL deletion strain, the polypeptide patterns of wild-type and mutant cells were analyzed. In the culture supernatants of cells grown at 37°C, the yields of secreted proteins appeared generally to be similar for the wild type and mutants (Fig. 3A), although some cell lysis seems to occur in the deletion strain. However, the supernatant of cultures grown at 20°C showed some differences in the polypeptide pattern (Fig. 3B), e.g., at 30 and 120 kDa. Also, some secreted but cell-associated proteins appeared to be absent even at 37°C in the deletion mutant, as judged by proteinase K accessibility (data not shown). Since some, but not all, extracellular proteins are absent in the yvaL deletion strain, it seems that YvaL is needed for the secretion of a specific subset of proteins. The size of the 30-kDa protein may correspond to that of mature endogenous β-lactamase. The β-lactamase activity was about two- to three-fold lower in the culture supernatant of strain DB104ΔyvaL than in that of strain DB104 (Fig. 4). Interestingly, the β-lactamase activity could be restored to normal levels by the expression of either B. subtilis YvaL or E. coli SecG. It is important to note that the E. coli β-lactamase present as an AmpR marker on the plasmids used is not expressed in B. subtilis (33). The β-lactamase activity of strain DB104 was the same with or without plasmid pPR111. These data strongly suggest that the B. subtilis yvaL deletion strain is impaired in the secretion of some proteins.

**YvaL does not complement the cold sensitivity of the E. coli secG null strain.** The secG disruption mutant E. coli KN370 shows a cold-sensitive phenotype (20). At 37°C, no growth defect is observed, while at 20°C, the strain is no longer able to form single colonies on agar plates. Upon induction with IPTG, plasmid pET304 expressing E. coli SecG was able to restore growth at the nonpermissive temperature of 20°C (Fig. 5). However, when E. coli KN370 was transformed with pET820 containing yvaL, no growth restoration was observed at the nonpermissive temperature, not even when the expression was induced by IPTG. On the other hand, growth was normal at the permissive temperature of 37°C. These data demonstrate that YvaL cannot functionally replace SecG in E. coli.

**DISCUSSION**

To facilitate functional studies on the precursor protein translocase of the gram-positive bacterium B. subtilis, we have cloned a homologue of SecG termed YvaL. The gene was identified on the basis of sequence similarity with its gram-negative counterpart. Although the overall identity is low, there is clear similarity between the YvaL and SecG proteins. Both proteins harbor two putative transmembrane segments that are connected via a glycine-rich loop. The YvaL protein is shorter than the SecG protein and lacks the carboxyl-terminal extension. This property is shared with other SecG homologues of gram-positive bacteria present in the databases. Like that of secG in E. coli (20), disruption of the yvaL gene in the...
chromosome of *B. subtilis* DB104 results in a cold-sensitive growth defect. However, this effect is mild compared to that in the *E. coli secc* null strain but is elevated when secretory stress is imposed by overexpression of the precursor form of α-amylase. The cold sensitivity can be overcome by expression of YvaL or SecG in *trans*, although growth is not restored to the level observed with the wild type only. Despite the care that was taken to disrupt only the *yvaL* open reading frame, the integration of the resistance marker could modulate the expression of the downstream genes and thereby affect physiology. It has been noted that the cold sensitivity of *E. coli* growth is strain dependent (10, 20). The reason for this is not entirely clear, but it may well relate to differences in growth physiology, secretion demand, and/or the level of translocase components in the various strains. Analysis of the profile of secreted proteins in wild-type *B. subtilis* and the *ΔyvaL* strain most notably reveals that two major proteins are absent in the latter strain. However, in the culture supernatant containing the secreted proteins, only certain polypeptides are affected. Therefore, it appears that deletion of *yvaL* does not result in a strong pleiotropic secretion defect but rather affects the secretion of a subset of proteins. No direct analyses of total secreted proteins has been performed with the *E. coli secc* null strain, but the in vitro translocation of the precursors *proOmpA* and *proOmpF*-Lpp demonstrates a clear difference in *SecG* dependence (19). Direct evidence that *SecG* and *YvaL* have the same function is provided by the observation that the secretion of β-lactamase in the *B. subtilis* *yvaL* strain is restored not only by expression of YvaL but also by that of SecG. On the other hand, YvaL cannot complement the *E. coli secc* null strain. In conclusion, our results demonstrate that *B. subtilis* YvaL is a functional homologue of *E. coli SecG*. It is concluded that the heterotrimetric organization of the integral membrane domain of the translocase is conserved well in bacteria.

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5. Breitling, R. Unpublished data.


9. Dowville, K., M. Leonard, L. Brundage, K. Nishiyama, H. Tokuda, S. Mi-


